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It is characteristic of Science and Progress that they continually open new fields to our vision—PASTEUR

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PRECURSORS TO THE FORMATION OF CREATININE BY BACTERIA

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Creatinine is a urinary excretory product from warm-blooded animals but the mode of its formation is not, as yet, completely understood. Folin (1905) determined that its production was independent of intake of food and protein and of non-protein nitrogen. Moreover, its daily output is normally constant. The amount of creatinine excreted per day is proportional to the muscular development of the individual as shown by Tracy and Clark (1914).

Since creatinine may be considered to be dehydrated creatine, it has been assumed that creatine is its forerunner, although findings here have been diverse (Folin, 1914 and Lyman and Bundy, 1917). In addition, arginine, choline, glycine-betaine and cystine have been advanced as precursors of creatine but final evidence of the effectiveness of anyone of these is lacking. It is possible that they may be only stimulants. The complexities of the animal body are such that evaluation of variables frequently becomes impossible and therefore it was decided to turn to simple organisms for our experiments, which have been done with certain bacteria.

Fitzgerald and Schmidt (1912) and Sears (1916, 1917) have demonstrated that bacteria can produce creatinine and the latter showed that *Proteus vulgaris* formed this compound from peptone. In the presence of glucose, with the peptone, the amount of creatinine appearing was increased. Because of the relatively simple structure of the bacterial media, control of variables is easier than in the body of an animal.

Creatinine may be related theoretically to acetic acid, glycine,

sarcosine, guanidine, methylguanidine, glycocyamine, arginine, urea or choline and certain of these were examined to determine their availability to act as precursors of creatinine when exposed to the growth activities of bacteria.

Procedure. Following our introductory experiments which were devoted to verification of the observations of Sears (1916, 1917), synthetic media were utilized. The common base for these media was Mueller's (1935) salt solution containing NaCl, K_2HPO_4, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$ dissolved in distilled water. This solution was sterilized in the Arnold Steamer. The following amino acids have been included: glycine, dl-alanine, dl-glutamic acid, d-arginine HCl, cystine, dl-phenylalanine, dl-tyrosine, dl-aspartic acid, dl-leucine, and these compounds were obtained from Amino Acid Manufactures at the University of California at Los Angeles and were stated to contain not more than 0.004 per cent of impurities. These substances were used in watery solutions and the concentration of hydrogen-ion was adjusted to 7.0. Sterilization was effected by the intermittent method. Media under test were compounded at time of use by addition of 0.25 cc. of Mueller's solution to 10 cc. of solution of the amino acid. Culture volumes usually were 60 cc.

The organisms were from stock cultures in the department of the University and had been previously isolated at various times.

Determinations of creatinine were made by the method of Jaffé (1886). A solution of creatinine chloride of concentration of 100 mlg. creatinine in 100 cc. of distilled water was standardized against one of potassium dichromate. In turn, this was diluted to suit subsequent tests. Saturated aqueous solution of picric acid was tested for purity by the method of Folin and Doisy (1917). A representative test was conducted in the following manner: Five cc. of culture solution plus 5 cc. of alkaline picrate stood for five minutes and then were diluted volumetrically to 25 cc. with distilled water. By a colorimeter this solution was compared with 5 cc. of the standard dilution of creatinine to which had been added 5 cc. of alkaline picrate. This, after standing for five minutes, in turn was diluted volumet-

rically to 25 cc. with distilled water. If culture solutions were turbid, they were centrifugalized to prevent interference with readings in the colorimeter.

EXPERIMENTAL

Experiment 1. Since Sears (1916) stated that *Staphylococcus aureus, Proteus vulgaris* and *Escherichia coli* produce creatinine in peptone water and that this creatinine is increased in amount in the presence of glucose, his work was duplicated with results

TABLE 1

Production of creatinine by three organisms listed

Amounts are expressed as milligrams in 100 cubic centimeters of media

	E. COLI		S. AUREUS		P. VULGARIS	
	3 days	14 days	3 days	14 days	3 days	14 days
Peptone water	4.8	5.2	3.5	3.3	5.1	5.0
Peptone water + 1 per cent glucose	5.6	6.0	4.0	3.4	6.5	6.8

TABLE 2

Production of creatinine by three organisms listed

Amounts are expressed as milligrams in 100 cubic centimeters of media. Incubation period was four days

	0.1 PER CENT GLYCINE	0.5 PER CENT GLYCINE
S. aureus	No growth	No growth
E. coli	0.40	0.40
P. vulgaris	0.45	0.50
Solution of sodium picrate	0.37	

indicated in table 1. Thus, the work of Sears (1916) has been confirmed and it is indicated that *P. vulgaris* is more active than *E. coli* or *S. aureus* in formation of creatinine.

Experiment 2. Since peptone contains a complex of amino acids, the next step was to consider one of the simplest of these, glycine, as a source for formation of creatinine when the three organisms used in experiment 1 were placed in contact with it. For this purpose solutions of glycine of 0.1 and 0.5 per cent concentration were utilized. Results are set forth in table 2.

Since sodium picrate, because of the color of its solution, gave an apparent reading of 0.37, it is obvious that a trace only of creatinine was produced in this test. These results indicated that further work was likely to deal with small differences and caused us in certain instances to modify the technique by adding standard creatinine solution to the unknown. The procedure of McCrudden, and Sargent (1916) as indicated below, was employed;

Unknown	*Standard*
5 cc. culture solution	5 cc. distilled water
5 cc. standard solution	5 cc. standard solution
5 cc. alkaline picrate	5 cc. alkaline picrate

Wait 5 minutes
Dilute to 25 cc. volumetrically
Read by colorimeter

In this experiment, glycine served as the sole source of C and N for the bacteria and of itself was insufficient for formation of creatinine.

Experiment 3. Glycine corresponds only to that portion of the molecule of creatinine which contains $\diagdown N—CH_2 \cdot COOH$. Since glycine can serve in such limited degree, it is likely that a precursor corresponding to the $HN{=}C—NH_2$ portion of the molecule of creatinine might be lacking. Urea might serve this purpose. Therefore various combinations of solutions of glycine, urea, ammonium sulphate and glucose were combined to constitute the series presented in table 3 with *Proteus vulgaris* to serve as the test organism. Ammonium sulphate was introduced into this series to serve as a control. To include ammonium carbonate would have incorporated an additional source of carbon and that was undesirable.

A portion of each of these formulae was kept uninoculated until the conclusion of the experiment and then was tested for possible presence of creatinine. Results were negative in each instance.

Since the results obtained by use of glycine, urea and glucose were so exceptional when compared to other units of the series,

the work with this particular combination was repeated. Readings were made at frequent intervals during incubation at 37°C. Creatinine appeared in a concentration of 1.6 milligrams per 100 cubic centimeters in twenty-four hours. At five days, it was 6.66 and at fourteen days, 5.0.

A mixture of glycine, urea and glucose produced the largest yield of creatinine by *Proteus*; while all other solutions used permitted formation of little or none of this by-product.

Experiment 4. Arginine contains the guanidine group and thus likewise may serve as a precursor to the formation of creatinine. Therefore the series presented in table 3 was set up, with arginine substituted for glycine. With the exception noted, all conditions were identical in the two experiments. The results of these tests are presented in table 4. It may be concluded that arginine is not so effective as is glycine for production of creatinine by *Proteus*. Moreover an examination of tables 3 and 4 indicates that in the presence of the amino acid and glucose, ammonium sulphate cannot replace urea for formation of creatinine.

TABLE 3

Production of creatinine by Proteus vulgaris when in presence of glycine, urea ammonium sulphate and glucose in various combinations

Results indicated as milligrams per 100 cubic centimeters

MEDIUM	4 DAYS	7 DAYS	14 DAYS
0.1 per cent glycine	0	0.1	0.1
0.1 per cent glycine + 1 per cent glucose	0.16	0.44	0.32
0.1 per cent glycine + 0.5 per cent urea	0.1	0.1	0.1
0.1 per cent glycine + 1 per cent glucose + 0.5 per cent urea	Not tested	13.3	3.0
0.1 per cent glycine + 1 per cent glucose + 0.5 per cent ammonium sulphate	Not tested	0.22	0
0.1 per cent glycine + 0.5 per cent ammonium sulphate	0.16	0.1	0.1
1 per cent glucose + 0.5 per cent urea	0	0	0
0.5 per cent urea (no growth)	0	0	0
0.5 per cent ammonium sulphate + 1 per cent glucose	0	0	0
0.5 per cent ammonium sulphate (no growth)	0	0	0

Experiment 5. Inasmuch as glycine and arginine may serve

with urea and glucose as sources for formation of creatinine, it was determined to examine other amino acids individually for like effect. These were incorporated with 0.5 per cent urea and 1 per cent glucose in the synthetic medium described previously and the results are shown in table 5.

TABLE 4

Production of creatinine from d-arginine HCl by Proteus

Concentrations presented as milligrams per 100 cc. medium

MEDIUM	3 DAYS	7 DAYS	14 DAYS
0.1 per cent d arginine HCl	0.1	0	0.15
0.1 per cent d arginine HCl + 1 per cent glucose	0.24	0.52	0.52
0.1 per cent d arginine HCl + 0.5 per cent urea	0	0	0
0.1 per cent d arginine HCl + 0.5 per cent ammonium sulphate	0	0	0
0.1 per cent d arginine HCl + 0.5 per cent urea + 1 per cent glucose	0.28	0.30	1.2
0.1 per cent d arginine HCl + 1 per cent glucose + 0.5 per cent ammonium sulphate	0	0	0

TABLE 5

Production of creatinine by Proteus from a series of amino acids in presence of glucose

Results are expressed as milligrams per 100 cc. of medium

AMINO ACID IN MEDIUM	3 DAYS	14 DAYS
0.1 per cent Glycine	4.17	4.0
0.1 per cent dl Alanine	3.46	2.0
0.1 per cent dl Glutamic acid	2.50	1.5
0.1 per cent Cystine	3.90	4.6
0.1 per cent Phenyl alanine	2.80	2.08
0.1 per cent dl Leucine	2.5	2.3
0.1 per cent dl Tyrosine	2.8	3.46
0.1 per cent dl Aspartic acid	2.08	3.9
0.1 per cent d Arginine HCl	2.30	2.0

Evidently creatinine can be formed by *Proteus* from all of these amino acids although glycine, cystine, aspartic acid and tyrosine are the most effective of the series.

Experiment 6. In the work described thus far, glucose has been the form of carbohydrate utilized. It was desirable to

determine whether another carbohydrate may be substituted for this hexose. A series identical to that given in table 3 except that a pentose, arabinose, replaced glucose was therefore inoculated with *Proteus*. All other conditions were similar. Final readings with the combination of glycine, urea and arabinose indicated the appearance of 0.22 milligram of creatinine per 100 cubic centimeters rather than 3.0 which were obtained when glucose was present. Subsequent work showed galactose to afford 40 to 65 per cent of the effectiveness of glucose. Maltose upon hydrolysis yields two molecules of glucose but when this

TABLE 6

Production of creatinine by bacteria in media described

Readings in terms of milligrams per 100 cc. of solution

ORGANISM	PEPTONE WATER	PEPTONE WATER + 1 PER CENT GLUCOSE	0.1 PER CENT GLYCINE + 1 PER CENT GLUCOSE + 0.5 PER CENT UREA	0.1 PER CENT D ARGININE HCl + 1 PER CENT GLUCOSE + 0.5 PER CENT UREA
Proteus	4.0	5.4	4.1	2.7
Proteus × 19	5.0	3.5	4.4	2.03
Alcaligenes	4.6	4.4	2.7	2.0
Mycobacterium phlei	3.3	4.1	1.2	2.05
Mycobacterium smegmatis	3.7	4.0	1.4	2.00
Bacillus subtilis	3.9	3.7	2.0	1.98
Aerobacter aerogenes	3.9	4.1	2.2	2.23
Escherichia communis	4.8	5.0	1.7	2.68
Escherichia communior	4.8	4.8	1.8	1.46
Escherichia lactici-acidi	4.0	4.1	1.6	2.05

disaccharide in turn replaced glucose in the media, the yield of creatinine was but 67 per cent of that produced by the monosaccharide. In further work arginine was substituted for glycine and arabinose for glucose. Again it was indicated that the presence of glucose is essential for production of the larger amounts of creatinine.

Experiment 7. Three organisms were considered in the first studies, while *Proteus* alone was used for those designed to determine production of creatinine. It was decided to amplify these findings by inclusion of additional forms. For this purpose, ten bacterial species were selected and with these was incor-

porated *Proteus vulgaris* to serve as a standard for comparison. Four media were utilized. Final readings of the concentration of creatinine and description of the media are presented in table 6. Incubation extended over a period of fourteen days at 37°C. These results indicate that creatinine in considerable concentration may be produced by a variety of bacterial species. With one exception it is demonstrated that more of the by-product is formed in the presence of peptone than with glycine alone and that in many instances, formation of creatinine is intensified by the presence of glucose.

Experiment 8. It was demonstrated in table 4 that small amounts of creatinine may be produced from arginine. The presence of urea was repeatedly shown to be necessary if con-

TABLE 7

The effect of manganese sulphate upon production of creatinine by Proteus

Concentrations noted as milligrams per 100 cc. of medium

MEDIUM	3 DAYS	14 DAYS
0.1 per cent d Arginine HCl + 1 per cent Glucose	0.24	0.52
0.1 per cent d Arginine HCl + 1 per cent glucose + 5 per cent of 0.1 Mol. $MnSO_4$	3.56	3.12
0.1 per cent Glycine + 1 per cent Glucose + 0.5 per cent urea	2.3	5.0
0.1 per cent Glycine + 1 per cent Glucose + 0.5 per cent urea + 5 per cent 0.1 Mol. $MnSO_4$	4.17	5.0

siderable concentrations of this compound are to appear. Arginine contains within its molecular structure two portions: a guanidine group HN = C(HN$_2$)(NH) and a moiety of urea = C(NH$_2$)(NH). The question then arises as to whether either of these is related to the production of creatinine by *Proteus*. Arginase breaks down arginine with the formation of urea and ornithine. Thus, it destroys the guanidine fraction. Hellerman and Perkins (1935) have demonstrated that salts of manganese and of cobalt

may serve as activators of arginase. It was determined to observe whether the presence of an activator of arginase would stimulate formation of increased amounts of creatinine.

Preliminary trials indicated that the cobalt would interfere but that manganese would not interfere with the reading of the reaction. Therefore, the following series presented in table 7 was set up and results as indicated after fourteen days incubation at 37°C. were obtained.

From these data it is evident that the destruction of the guanidine portion of the molecule of arginine with resultant formation of urea gives rise to the appearance of increased concentration of creatinine when exposed to the enzymatic action of *Proteus*. Additional evidence regarding the participation of urea in formation of creatinine is adduced.

DISCUSSION AND CONCLUSION

Creatinine can be produced from peptone by a variety of bacteria but the peptone must be considered as a complex structure involving many factors. Further consideration has proven that creatinine may be formed through bacterial action from various amino acids when glucose is present. In seeking for its precursors, creatinine has been treated as a compound containing two critical portions which are (1) acetic acid and (2) guanidine. It has been shown that glycine, urea and glucose when under the influence of *Proteus vulgaris* give rise to considerable concentrations of creatinine. Other amino acids than glycine supply either the acetic acid moiety or urea necessary for the reaction. Added evidence regarding the importance of urea is presented by treatment of arginine by an activator of arginase. For production of creatinine by *P. vulgaris* urea may not be substituted by a salt of ammonium. The hexose glucose is the form of carbohydrate most readily utilized by the organism in the course of creatinine production. The precursors of creatinine as produced by *Proteus* are acetic acid moiety and urea or glycine and urea.

We are indebted to Dr. V. R. Goddasd for her advice in this work.

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A COMPARATIVE STUDY OF THE USE OF VARYING CONCENTRATIONS OF AGAR IN THE TEST MEDIUM USED TO DETECT CONTAMINANTS IN BIOLOGIC PRODUCTS[1]

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INTRODUCTION

Previous reports on the bactericidal action of antiseptics used in biologic products (Rosenstein and Levin, 1935; Falk and Aplington, 1936) showed that, when a given organism was inoculated into both broth and agar media, growth sometimes appeared in the agar and not in the broth and vice-versa. These findings could not be attributed to bacteriostatic action caused by insufficient dilution of the antiseptic present, since identical results were at times obtained in the control tubes containing no antiseptic. It was therefore indicated that neither broth nor agar used alone could be relied upon to detect organisms which may be found as contaminants (staphylococci, diphtheroids, and *Pseudomonas pyocyaneus*) in biologic products. These observations were later corroborated by Eldering and Kendrick (1936).

A semifluid medium, 0.1 per cent agar, was recommended by Hitchens (1921) for the detection of bacterial contaminants, either aerobic or anaerobic, particularly in substances containing a high percentage of antiseptic. While this medium has not attained the universal use that 0.03 per cent glucose broth has for testing the sterility of biologic products, it has been used in conjunction with this broth by Wadsworth (1927) and others.

[1] Presented at the Thirty-Ninth General Meeting of the Society of American Bacteriologists, Washington, D. C. December 30, 1937. Journ. Bact. **35**: 1, 75.

More recently, Spray (1936) reported that anaerobes previously considered difficult to cultivate, grew well on a semisolid medium without a seal. We have not found, however, any extended experimental or statistical studies which demonstrated that a semifluid, semisolid, or solid medium would be preferable to broth for the detection of the more common forms of bacterial growth, or as a substitute for the regular 0.03 per cent glucose broth for testing the sterility of biologic products. It seemed, therefore, advisable to make such a study, particularly since the use of two very different types of medium is obviously undesirable in any laboratory concerned with large scale production of biologic products.

EXPERIMENTAL METHOD

The efficiency of different concentrations of agar in the medium used for the detection of bacterial growth was evaluated by (1) adding organisms directly to the test media; (2) adding organisms to typical biologic products, containing preservative, and subculturing to the various test media; and (3) adding a biologic product known to have a bacterial contaminant to the various test media.

Concentrations of agar in the test media varied from 2.0 to 0.001 parts of agar per hundred (or one to 2000 parts of agar per 100,000). The media containing from one to 0.001 parts of agar per hundred were prepared by diluting 2 per cent biologic-products agar with sufficient biologic-products broth (containing 0.03 per cent glucose) so that the desired concentrations of agar were obtained. The method of preparing these basic media has been described in previous papers (Rosenstein and Levin, 1935; Falk and Appington, 1936). The media, made up in 300 cc. batches, were tubed aseptically in 6″ x $\frac{5}{8}$″ tubes, 10 cc. being delivered into each tube. Immediately before use the test medium was heated at 100°C. in the Arnold sterilizer for one hour, followed by quick cooling to 45°C. As in our previous studies, the test organisms were types which may be encountered as contaminants in biologic products. In fact the strains used were actually isolated from such products over a period of years.

In the experiments to be described here, the staphylococci, diphtheroids, and *P. pyocyaneus* previously used were supplemented by hay bacilli, streptococci and *Escherichia coli*. Eighteen-hour broth cultures, containing approximately 100,000,000 organisms per cubic centimeter (colony counts varied from 60-000,000 to 250,000,000), of the above-mentioned organisms were diluted with broth so that the 10^{-4} dilution contained approximately 10,000 organisms per cubic centimeter, the 10^{-5} dilution 1000 organisms per cubic centimeter, the 10^{-6} dilution 100 organisms per cubic centimeter and the 10^{-7} dilution 10 organisms per cubic centimeter. A plate count was made to determine the exact number of organisms present in every experiment. One-tenth of a cubic centimeter of the appropriate dilution was then added to each of the various test media directly or to typical biologic products, which were subcultured to the test media. These tests were then incubated at 36°C. for seven days, during which time observations were made daily. All tests were carried out in duplicate, each complete series of tests being repeated at least once. The identity of organisms in tubes showing growth was checked by smears stained by Gram's method.

EXPERIMENTAL RESULTS

Typical results are given in tables 1 to 3. In the first series of experiments, in which organisms diluted with broth were used, there was a striking variation in the rate and character of the growth of the organisms in the various test media. These differences were apparently dependent on the percentage of agar present. Growth was inhibited when the percentage of agar was too high as well as when the medium was too fluid (table 1). In all cases, when the concentration of agar in the medium was between 0.06 and 0.50 parts per hundred, growth was most luxuriant. This fact became increasingly evident as the size of the inoculum decreased. In fact, when the medium contained concentrations of agar between 1.0 and 0.12 per cent, the presence of very few organisms could be demonstrated, whereas negative results were obtained in the media containing either

TABLE 1

The effect of different concentrations of agar on the growth of organisms when varying inoculums are used

7 day readings

AGAR	HAY BACILLI			STAPHYLOCOCCI			P. PYOCYANEUS			DIPHTHEROIDS		
	DILUTED			DILUTED			DILUTED			DILUTED		
	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}
per cent												
2.0	++	++	++	++	++	+	+	+	−	−	−	−
1.0	++	++	++	++	++	+	++	+	+	+	+	+
0.50	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+	+
0.25	++++	++++	++++	++++	++++	+++	++++	++++	+	+++	+++	++
0.12	++++	++++	++++	++++	++++	−	++	++	+	++	++	+
0.06	++++	++++	++++	++++	++	−	++	++	−	++	++	+
0.03	+++	+++	++	++	++	−	+	+	−	+	+	+
0.015	++	++	+	++	++	−	+	+	−	+	+	+
0.008	++	++	+	++	++	−	+	+	−	+	+	+
0.004	++	+	+	++	++	−	+	+	−	+	+	+
0.002	++	+	+	++	+	−	+	+	−	+	+	+
0.001	+	+	+	+	+	−	+	+	−	+	+	+
0.000	+	+	+	+	−	−	+	+	−	+	+	+

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

more or less agar in many cases. As might be expected, individual preferences for definite concentrations of agar in the medium were shown by the different test organisms. For example, staphylococci inoculated in small numbers did not grow in a medium containing less than 0.25 per cent agar, diphtheroids if the medium contained more than 1 per cent agar, *P. pyocyaneus* if more than 1 per cent or less than 0.25 per cent agar was present; hay bacilli on the other hand, showed growth in all the test media, but this growth was most luxuriant when the medium contained between 0.06 and 0.25 per cent agar.

To determine the practical application of these results, another series of experiments was carried out in which actual routine working conditions were more nearly approximated. Results obtained when test organisms were added to typical biologic products are shown in table 2. Typical products included a bacterial vaccine, an antitoxic globulin, and an antibacterial serum. Eighteen-hour broth cultures of each of the organisms were diluted with broth so that each cubic centimeter contained 10,000 organisms; 0.5 cc. of this dilution was then added to sterile 5 cc. samples of the above named products. Subcultures were made to the various test media after the culture had been in contact with the product for thirty minutes, since in most cases the preservative present might be expected to destroy the test organisms on continued standing. Again as in our first experiments, maximum growth was obtained when the concentration of agar in the medium varied from 0.06 to 0.50 per cent. The exact optimum concentration of agar needed, as before, depended on the type organisms present. The intermediate environment to which the organisms had been subjected, also, seemed to influence the concentration of agar in the medium which produced the most luxuriant growth on subculture. Organisms added to the bacterial vaccine corresponded most closely to the broth control, most luxuriant growth occurring in media containing from 0.12 to 0.5 per cent agar. On the other hand, organisms added to the antitoxic-globulin needed a more fluid medium, that is, one containing from 0.12 to 0.06 per cent of agar; and organisms added to the bacterial serum

TABLE 2

The effect of different concentrations of agar on the growth of organisms

Readings made after incubation at 36°C. for 96 hours

AGAR	HAY BACILLI				STAPHYLOCOCCI				P. PYOCYANEUS			
	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin	Broth control	Bacterial vaccine	Antibacterial serum	Antitoxic-globulin
per cent												
2.0	++	++	++	+	+	+	−	+	++	+	+	+
1.0	+++	++	+++	++	++	++	+	++	++	+	+	+
0.5	++++	+++	+++	+++	++	+++	++	+++	+++	+++	++	+++
0.25	++++	++++	++	+++	+++	+++	+++	+++	++++	++++	+++	+++
0.12	++++	++++	−	++++	++++	++++	++++	++++	++++	++++	++++	++++
0.06	+++	+++	−	++++	+++	+++	++	++++	+++	+++	+++	++++
0.03	+++	++	−	+++	++	++	−	+++	++	++	+	+++
0.000	++	+	−	++	++	+	−	++	++	+	+	++
	DIPHTHEROIDS				STREPTOCOCCI				E. COLI			
2.0	++	+	+	+	+	+	+	+	+	+	+	+
1.0	++	++	+	++	++	++	++	++	++	++	++	+
0.5	++++	+++	++	+++	+++	+++	+++	++	+++	+++	++	+++
0.25	++++	++++	+++	+++	++++	+++	+++	++	++++	++++	+++	+++
0.12	++++	++++	−	++++	++++	+++	++++	++++	++++	++++	++++	++++
0.06	++	+++	−	++++	+++	++	++	++++	+++	+++	+	++++
0.03	++	++	−	+++	++	++	−	+++	++	++	−	+++
0.00	++	+	−	++	+	+	−	++	++	+	−	++

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

TABLE 3

The comparative efficiency of different concentrations of agar in the sterility test medium when gram-positive aerobic bacilli were present in a known contaminated product

AGAR	DAYS INCUBATED AT 36°C.														
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
per cent															
2.0	−	++	++	−	++	++	−	++	++	−	++	++	−	++	++
1.0	+	+++	+++	+	+++	+++	+	+++	+++	+	+++	+++	+	+++	+++
0.5	++	+++	+++	++	+++	+++	++	+++	+++	++	++++	++++	−	++++	++++
0.25	++	++++	++++	++	++++	++++	++	++++	++++	++	++++	++++	+++	++++	++++
0.12	++	++++	++++	+++	++++	++++	−	+++	++++	+++	++++	++++	+++	++++	++++
0.06	++	+++	+++	++	+++	+++	++	++	+++	++	+++	+++	++	+++	+++
0.03	++	++	+++	++	+++	+++	−	++	+++	+	+++	+++	++	+++	+++
0.00	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−
	Vial 1			Vial 2			Vial 3			Vial 4			Vial 5		

+, scant growth; ++, moderate growth; +++, good growth; ++++, luxuriant growth; −, no growth.

* Growth observed in only one of two similarly inoculated tubes.

seemed to favor the media with concentrations of agar varying from 0.12 to 0.5 per cent, often giving negative results if less than 0.1 per cent agar was present. However, irrespective of the type of organism or its environment, optimum growth was regularly obtained in the media containing from 0.12 to 0.25 per cent of agar.

Soon after the completion of these experiments, we were able to secure several vials of a product which had become contaminated with a gram-positive aerobic bacillus in the process of manufacture. This particular product had shown growth in only one broth fermentation tube out of the twenty-five inoculated in routine sterility tests. The poured agar plates also showed no growth. For the experimental series of tests, 0.1 cc. portions from each vial were added to each of two tubes containing 10 cc. of test medium. Results of these tests are given in table 3. Again, only one of the ten broth tubes inoculated showed growth, while good growth was observed in every tube in which the medium contained between 1.0 and 0.1 per cent of agar. This experiment also demonstrated that the time at which growth was first observed in any one tube was influenced by the concentration of agar present. Good growth was first noticed in 48 hours in media containing concentrations of agar varying from 0.12 to 0.25 per cent, but it required at least 96 hours to show the same type of growth in media containing more than 0.25 per cent agar or less than 0.12 per cent agar, and as much as seven days when broth alone was used.

PRACTICAL APPLICATION

The experiments which have been described all showed that bacterial growth could be readily detected in media containing between 0.1 and 0.2 per cent agar. It therefore seemed advisable to use this medium in conjunction with the regular biologic-products broth for the routine testing of sterility of biologic products. All sterility tests were carried out following the recommendations of the National Institute of Health, with the exception that one half of the volume of product to be tested was added to the broth in fermentation tubes, while the re-

mainder was added to the 0.1 per cent agar medium. For routine purposes, the medium was dispensed in 7″ x 1″ metal capped tubes, each tube containing 35 cc. of medium. This medium received the same preparatory treatment as the biologic-products broth in fermentation tubes, that is, heating at 100°C. for one hour in the Arnold sterilizer, within two hours before use, followed by rapid cooling to 45°C. As has been pointed out by Wadsworth (1927), it is of the utmost importance that the 0.1 per cent agar medium be clear, so that individual colonies may be observed throughout the medium as

TABLE 4

The comparative efficiency of biologic-products broth and 0.1 per cent agar for the detection of contaminants in biologic products

LOT	B.P. BROTH			0.1 PER CENT AGAR		
	Tubes inoculated	Tubes contaminated	Contamination	Tubes inoculated	Tubes contaminated	Contamination
			per cent			*per cent*
a	42	42	100	70	53	76
b	30	3	10	30	24	80
c	60	17	28	100	76	76
d	60	38	63	100	14	14
e	72	8	11	120	71	59
f	92	1	0.9	130	34	26
g	36	0	0.0	60	9	15
h	100	2	2.0	120	19	16
i	36	1	2.8	60	5	8.3
Total.....	528	112	21.2	790	305	38.6

well as growth on the surface. Cloudy media necessitate subculture or the preparation of smears, in which case, any dead organisms present may cause confusion. To facilitate the inoculation and reading of tests, whenever possible, the broth and 0.1 per cent agar tubes were arranged in parallel rows in monel metal racks constructed for this purpose. Careful statistics were kept on the results obtained with these two media in the routine testing of the sterility of biologic products for a period of eight months. The comparative efficiency of the biologic-products broth and 0.1 per cent agar in the tests which showed bacterial contamination are given in table 4. Each lot showing

bacterial growth was retested at least once. The contaminating organisms in these lots were Gram-positive aerobic bacilli. The adoption of the mixture of phenol and merthiolate, as a preservative in most biologic products has markedly reduced the contaminations caused by *P. pyocyaneus*, diphtheroids and staphylococci, but this mixture is not effective against sporebearing organisms of the hay bacillus group. In general, growth was demonstrated in approximately twice as many of the 0.1 per cent agar tubes as in the biologic-products broth tubes. If only a light contamination was present, few if any broth tubes showed growth, in contrast to from 8 to 26 per cent of the tubes containing 0.1 per cent agar. In fact, in one of these nine lots showing growth, the product would have been released as sterile had only broth fermentation tubes been inoculated. On the other hand, where the contamination was extensive, growth was observed in both types of medium, but in most cases growth appeared earlier and was detected in more of the 0.1 per cent agar tubes. This was, however, reversed in two cases, one in which slightly more of the broth tubes showed growth, and another in which the apparent difference was more significant, only 14 of the 0.1 per cent agar tubes showing growth in contrast to 63 per cent of the broth tubes. We have also observed that the inclusion of the 0.1 per cent agar medium offers a great advantage over the exclusive use of biologic-products broth in fermentation tubes in that this medium contains just enough agar to permit the formation of separate colonies, thus permitting a quantitative estimate of the extent of the contamination present.

SUMMARY AND CONCLUSIONS

These experiments demonstrate the value of using a medium containing small percentages of agar (0.06 to 0.25 per cent) for the detection of bacterial growth. This applies even to such common forms as hay bacilli and staphylococci, which are ordinarily considered easy to cultivate. Since little difference in growth could be detected in media containing between 0.1 and 0.25 per cent agar, 0.1 per cent agar was chosen for com-

parative tests with 0.03 per cent glucose biologic-products broth for the routine testing of biologic products for sterility. (It is of interest as has been mentioned in the introduction to note that this medium was suggested by Hitchens in 1921, but did not obtain the universal acceptance that biologic-products broth did.) The results of our routine tests with 0.1 per cent agar have confirmed our experimental findings, as well as confirming and extending those of Hitchens (1921) and Spray (1936). The adoption of a semifluid medium as a standard means of detecting and giving a quantitative estimate of the extent of bacterial growth in biologic products can therefore be recommended. Applications to other materials and procedures involving bacterial growth are also indicated.

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DOUBLE-ZONE BETA-HEMOLYTIC STREPTOCOCCI

Their Cultural Characteristics, Serological Grouping, Occurrence and Pathogenic Significance

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The appearance of double-zone beta-hemolytic streptococci in blood agar has been described in a previous publication (Brown, 1937b). If this striking appearance were one which might be displayed by various hemolytic streptococci it would be nothing more than an interesting phenomenon. Since it is found to be correlated with certain cultural reactions and serological groupings it assumes systematic and diagnostic significance. Its practical value is enhanced because it often enables these streptococci to be recognized in the primary blood-agar plate, even before pure cultures have been obtained.

This report embraces a study of 188 strains, of which 138 are from human sources and 50 of animal origin. Eighty-six strains were received from other workers (R. C. Lancefield, H. Plummer, R. B. Little, G. J. Hucker, L. Kirschner, J. M. Murphy, W. D. Frost, J. M. Rosell, E. I. Parsons). Of these strains 55 were from Lancefield, including 33 isolated by Ronald Hare, 6 by M. H. Dawson and 10 by W. N. Plastridge, the remaining 102 strains were isolated in the author's laboratory.

As indicated in a previous publication (Brown, 1937a), three fermentative groups of double-zone beta-hemolytic streptococci were found with reference to lactose and salicin: lactose−, salicin+; lactose+, salicin+; and lactose+, salicin−. All strains hydrolysed sodium hippurate and fermented glucose, sucrose and trehalose; none fermented mannitol, raffinose, inulin

or sorbitol. The final pH in glucose broth was 4.3 to 4.9; all but three of the strains within 4.4 to 4.6. Eight strains, including representatives of the three fermentative groups, were tested for the fermentation of other substances and all gave the following results: levulose, galactose, maltose, glycogen and dextrin fermented; inositol, adonitol, dulcitol, xylose, arabinose, rhamnose and esculin not fermented.

For the determination of the fermentation reactions of streptococci, three precautions are regarded as essential: the basic medium must be one which supports good growth of the organisms in the absence of fermentation; the test sugars, other than glucose, must not be sterilized in the medium; the cultures must be incubated sufficiently long to detect slow fermentations. In these studies, the basic medium has been broth made from fermented meat infusion to each tube of which 3 or 4 drops of sterile ascitic fluid were added aseptically. The carbohydrates (5 or 10 per cent solutions in distilled water) were sterilized in the autoclave or by filtration and added aseptically to the sterile fermented broth. Before inoculation the media were incubated to determine sterility. The cultures were incubated for at least five days before final readings were made by determining the pH of the glucose broth cultures and noting the color of the brom-cresol-purple which was used as an indicator in the other sugar media. The importance of the above precautions was demonstrated by the study of a number of strains received from other laboratories and reported in the literature as fermenting lactose when tested in Hiss serum water. When these strains were retested by us, they fermented lactose which had been added to Hiss serum water before sterilization, but in Hiss serum water and in fermented infusion broth with lactose added aseptically, there was no fermentation, although good growth occurred.

The hydrolysis of sodium hippurate was determined in infusion broth containing 1 per cent of sodium hippurate. Before testing with ferric chloride reagent, distilled water was added to the previously marked tubes to replace water lost by evaporation during storage and incubation. By observing this precaution, and by using a properly balanced and tested reagent, we have never failed to obtain definite and consistent results.

Twenty-seven strains of double-zone hemolytic streptococci were inoculated onto 40-per cent bile agar. In comparison with

hemolytic streptococci of serological groups A and C most of the strains grew well although a few showed only meagre growth. Our impression is that medium containing 40-per cent bile is not sufficiently differential for final differentiation of the streptococci.

Twenty-one of the double-zone strains of human and bovine origin and representing all of the three fermentative groups were tested for fibrinolytic activity against human plasma by the technique of Tillett and Garner (1935). None was fibrinolytic.

White mice were inoculated intraperitoneally with 0.5 cc. of over-night ascitic-fluid broth cultures of most of the strains. The results are shown in table 1. The mice were observed for a week after inoculation. When death occurred it was usually

TABLE 1

Results of injection into mice

SOURCE	FERMENTATIVE GROUP	MICE	
		Survived	Died
Human	Lactose −, Salicin +	17	61
	Lactose +, Salicin +	15	36
	Lactose +, Salicin −	1	5
Bovine	Lactose +, Salicin +	20	5
	Lactose +, Salicin −	16	1
Guinea pig	Lactose −, Salicin +	1	2
Rabbit	Lactose −, Salicin +		1
Horse	Lactose +, Salicin +	1	

within 48 hours. If the inoculation of mice with such large amounts of culture is of any significance, it would appear that strains of the lactose−, salicin+ group are more frequently pathogenic for mice than are the other strains. It also appears that the human strains of whatever fermentative group are more frequently pathogenic for mice than are the bovine strains.

When hemolysin tests were carried out by adding 0.5 cc. of a 5-per cent suspension of washed rabbit-blood erythrocytes to 0.5 cc. of young serum broth cultures and incubating for two hours, the double-zone hemolytic streptococci were variable in their reactions, i.e., different strains produced various amounts of hemolysis, from slight to complete. Most strains produced

moderate hemolysis. The amount of hemolysis was independent of the source or fermentation reactions of the strains. These streptococci are therefore less consistently hemolytic by this test than are strains of serological groups A and C.

All of the 188 strains of double-zone beta-hemolytic streptococci studied have been found to belong to Lancefield's serological group B by the precipitin test. Seventy-five of these strains had been grouped by other investigators (Lancefield and Plummer) using the Lancefield technique (1933). Most of them, and all of the remaining strains, were also grouped by us, using the microscopic technique of Brown (1938). We have also encountered a few hemolytic streptococci of bovine origin falling into group B which did not produce double zones in our media but which were otherwise culturally like the double-zone strains. It may be said, therefore, that according to our experience to date, all double-zone beta-hemolytic streptococci belong to serological group B but that there may be a few strains of group B which do not produce double zones under the conditions which we have employed.

Sixty-seven of the double-zone strains have been serologically "typed" by Lancefield or Plummer. No close correlation of source, fermentative characters and serological types was apparent. Further study will be necessary to determine whether there is any correlation.

In table 2 the specific sources and the fermentative groups of all of the double-zone beta-hemolytic streptococci studied are listed. In human beings the throat and the genito-urinary tract may be regarded as their habitat. Of the 22 throats from which strains were isolated, only two were described as "sore" and it is not certain that the condition of these two was due to the double-zone streptococci. Nothing is known of two of the tonsil cases and the other two tonsil strains were recovered from removed tonsils, one patient having complained of frequent sore throat. These were the only strains found in the examination of 100 pairs of removed tonsils. Again, the relation of the double-zone streptococcus to the clinical condition is unknown. Most of the 46 vaginal strains were taken post-partum from women with

afebrile puerperium. One was from a woman with a post-partum temperature of 100°F. but without other evidence of infection reported. A few strains were found ante-partum during routine examinations. Of the 32 urine cultures all were from cases of

TABLE 2

Sources and fermentative groups of human strains studied

SOURCE (HUMAN)	LACTOSE− SALICIN+	LACTOSE+ SALICIN+	LACTOSE+ SALICIN−
Throat	7	9	6*
Tonsils	2	2	
Vagina	20	26	
Urine	26	6	
(Probably derived from the mouth or throat)			
Sinus	1		
Lung	10	4	
Heart blood, 15116	1		
Neck abscess		1	
(Probably derived from the genito-urinary tract)			
Pelvic abscess	2		
Blood (abortion) during life		1	
Knee	1		
Heart blood, post-mortem	6		
Peritoneum	2	1	
(Infections of obscure origin)			
Peritoneum		1	
Ventricular fluid (hydrocephalis)	1		
Gangrenous leg	1		
Source unknown (Lancefield 090)		1	

* These six strains were obtained from Plummer and are representative of those found by her in the throats of children in Institution B.

urinary tract infection (cystitis, pyelitis, urethritis, prostatitis). Three-fourths of them were from women. In sixteen instances no other organism than the double-zone beta-hemolytic streptococcus was obtained in culture and the latter was often present in large numbers. There seems no doubt that these streptocci

may be a cause of such infections of the urinary tract. It is to be noted that most of the strains recovered from urine failed to ferment lactose.

Hare and Maxted (1935) were unable to find hemolytic streptococci of serological group B in the stools of 150 women examined. Smith and Sherman (1938) reported six strains isolated from human feces. The normal throat and the vagina of some individuals appear to be the habitats of double-zone beta-hemolytic streptococci. From these sources infection may occur, as indicated in table 2. Some of the cases may be worthy of discussion.

Fourteen strains were obtained from lungs at autopsy but always mixed with other organisms. The pathogenic rôle of the double-zone streptococci is doubtful in these cases.

Case 15116 was one of typhoid fever of several weeks duration (male, age 36). At autopsy there was no perforation of the intestine and no peritonitis but there were found purulent sinusitis, otitis media and a deep ulceration of the vocal cord extended into the cartilage. At the base of the ulcer were masses of Gram + cocci. From the heart blood at autopsy there were obtained large numbers of double-zone hemolytic streptococci and a smaller number of colon bacilli. It seems probable that death was due to terminal blood stream infection by double-zone streptococci derived from the throat.

The pelvic abscesses were in women and in one of them the double-zone streptococcus was found in pure culture.

In the fatal case of septicemia following abortion 100 double-zone hemolytic streptococci per cubic centimeter of blood were found in pure culture during life.

From an arthritis of the knee, double-zone hemolytic streptococci in large numbers were found in pure culture. A similar organism was found in small numbers in the blood stream. There had been a chronic pyuria and the patient was diabetic. Death followed operation on the knee but the cause of death was not determined.

In six other cases double-zone hemolytic streptococci were cultured from the heart blood post-mortem. Three of the cases were infants in the blood of whom only the streptococci were found; one lived only 24 hours after birth, one four days with hemorrhagic disease of the new-born, and one was born prematurely but lived for one month. Infection may have been contracted from the mothers at birth. The

other three cases were adults with pathological findings which suggest possible infection from the genito-urinary tract (carcinoma of the bladder, pyelonephritis and cystitis, arsphenamine poisoning with salpingitis and cysts in the ovaries and cervix uteri).

In three cases peritoneal infection appeared to be correlated with genito-urinary lesions (abscess of the broad ligament, carcinoma of the prostate and adenoma of the kidney, pyelonephritis and cystitis).

In three cases the infections were of obscure origin and in the ventricular fluid of one of them the double-zone streptococci were found in pure culture.

Reference to table 3 shows that 80 of the strains isolated from human beings were of the lactose − group and that 18 of the

TABLE 3

Strains from all sources

SOURCE	LACTOSE − SALICIN +	LACTOSE + SALICIN +	LACTOSE + SALICIN −
Human	80	52	6*
Bovine		27	18
Guinea pig	3		
Rabbit	1		
Horse		1	
Totals	84	80	24

* These six strains were obtained from Plummer and are representative of those found by her in the throats of children in Institution B.

strains from cow's milk were of the salicin − group. To date, lactose − strains have not been reported from bovine sources and salicin − strains have not been reported from pathological conditions in man. It is obvious that the human infant may have an opportunity to acquire double-zone hemolytic streptococci at birth. There certainly is no evidence that the lactose − strains found in man are derived from cows milk. Judging by their history (Plummer, 1934) the 6 salicin − strains isolated from children's throats may have been of bovine origin. If the human throat may serve as a carrier of salicin − strains derived from milk, there appears no reason why it may not also carry lactose +, salicin + strains from milk, but it does not follow that all such strains are

from milk. Strains fermenting both lactose and salicin were from both human and bovine sources and appear to be alike by current methods of study, but to speculate as to whether such strains found in man were originally bovine seems as fruitless as would be an effort to determine whether the colon bacilli originated in the intestinal tract of the cow or man. The pathogenic rôle of these streptococci for man bears a certain similarity to that of the colon bacilli. In the blood stream they may produce septicemia; in the abdomen, peritonitis; in the urinary tract, cystitis or pyelitis; they may infect sinuses or produce localized abscesses in various parts of the body. The invasion of the blood stream by double-zone hemolytic streptococci is not always fatal. We have on record a case of puerperal sepsis (retained placenta) in which the streptococci were found in the blood for nearly two months, followed by recovery. The prognosis for infections with these streptococci is more favorable than for infections with group A streptococci. The more than sixty strains in our collection from human autopsies and pathological conditions gives undue prominence to their pathogenicity. From 2000 autopsies cultured in this laboratory there were isolated 307 strains of beta-hemolytic streptococci and only 45 of these were double-zone streptococci. Very few of the latter could be suspected as the cause of death, and in many instances they were of no greater significance than other terminal or postmortem invaders. In other than a hospital population one might collect a large number of such strains without encountering pathological conditions. These streptococci are often harbored by normal individuals. They appear to be opportunist pathogens, as are many other organisms normally harbored by man, and there is no evidence that they cause contagious disease or are of epidemic significance.

There is evidence that the lactose +, salicin + and the lactose +, salicin − double-zone beta-hemolytic streptococci are of epizootic importance. Of the 45 strains from cow's milk, twenty-seven, including all but one of the salicin − strains, were from individual cows with clinical mastitis; five were received from other laboratories, labeled *Streptococcus mastitidis*; and the re-

maining fourteen were from mixed milk or were simply labeled "from milk" when received. Both the lactose +, salicin + and the lactose +, salicin − strains are causes of mastitis and probably are commonly transmitted from cow to cow during milking. R. B. Little (1937a) has shown that experimental mastitis may be produced with small amounts of culture introduced beyond the sphincter without injury to the teat, or by allowing the teat to aspirate culture or contaminated milk from a dish held in the hand (1937b). By the former method, Little (1938) produced mastitis in first-calf heifers with six double-zone beta-hemolytic streptococci isolated by us from human sources. See table 4.

In view of the above results there must be entertained the possibility of the infection of cows with double-zone streptococci

TABLE 4

Strains used for inoculation of cows udders

STRAIN	SOURCE	SEROLOGICAL		FERMENTATION	
		Group	Type	Lactose	Salicin
Nr	Septicemia following abortion	B	III	+	+
Fy	Normal throat	B	II	+	+
T78	Removed tonsil	B	Unclassified	+	+
BB	Blood of new-born infant	B	Ib	−	+
Gn	Normal throat	B	Ib	−	+
Py	Urine: cystitis	B	III	−	+

of human origin by the hands of milkers. That it may not happen frequently is indicated by the relative infrequency of the infection of the udder by group A hemolytic streptococci and by the absence, to date, of reports of the isolation of lactose −, group B hemolytic streptococci from the udder. It would appear that the human carrier needs to be considered, in attempts to eradicate mastitis from herds of cattle.

Among the double-zone beta-hemolytic streptococci studied were five from other animals than cows (table 3). Three strains were from guinea pigs, two from infected eyes and one from a foot, reported by Parsons and Hyde (1928). One strain was from a rabbit (Lancefield's strain K 158 A) used for testing vaccine virus. The above four strains were lactose −, salicin +.

The fifth strain was lactose +, salicin + and was isolated in 1908 from a horse with the diagnosis of "strangles." The stock strain has not been subjected to animal passage since its isolation. No other details of the case are known.

The lactose + double-zone beta-hemolytic streptococci would fall into "Group Ib" mastitis streptococci as described by Minett (1935), the *Streptococcus agalactiae* of some authors and the *Streptococcus mastitidis* of others. The tendency of some is to recognize both hemolytic and non-hemolytic varieties under these species, since both are common causes of mastitis and some of the non-hemolytic strains are reported to belong to serological group B and to be similar to the hemolytic strains except with regard to hemolysis. However, only a limited number of cultural tests have been employed and the discovery of other tests may reveal significant differences as was the case with *Streptococcus pyogenes* and some of the animal strains of serological group C previously indistinguishable from *Streptococcus pyogenes*. Frost, Gumm and Thomas (1927) proposed the name *Streptococcus asalignus* for the salicin − hemolytic streptococcus from milk which probably is identical with our lactose +, salicin − double-zone hemolytic streptococcus. Frost and Engelbrecht (in press) propose the name *Streptococcus mastitidis* for the hemolytic lactose +, salicin + mastitis streptococcus and the name *Streptococcus agalactiae* for the otherwise similar non-hemolytic mastitis streptococcus. There is equally good reason for giving a specific name to the lactose −, salicin + double-zone beta-hemolytic streptococcus belonging to serological group B. It would be a convenience to be able to designate these cultural entities by specific names, since the use of more general terms, the letters of the alphabet and even the Roman and Arabic numerals is becoming confusing and is meaningless without reference to the individual author cited. However, this is a situation which probably should be tolerated until more is known about the meaning of bacterial species in a rapidly developing field of study. The inclusion of more than one species in a single serological group need be no deterrent, since most authors recognize several species of hemolytic streptococci in serological groups A, C and

D. Neither need the finding of similar type antigens in bacteria of different species or genera be disturbing (recently discussed by Lancefield, 1938). It would appear that a species of *Streptococcus* need not be limited to a single serological type but should not transcend a serological group. I question whether streptococci of the same serological group and type need be placed in the same species if they show stable cultural differences.

No instability nor variability has been noted in the strains of double-zone beta-hemolytic streptococci in our possession although some of them have been under observation for twenty-five years.

SUMMARY AND CONCLUSIONS

Double-zone beta-hemolytic streptococci are found in the throats and vaginae of many normal persons. They are also a common cause of mastitis in cows.

These streptococci fall into three fermentative groups: lactose −, salicin +; lactose +, salicin +; and lactose +, salicin −.

All of the 188 strains examined belong to Lancefield's serological group B by the precipitin test.

In man, the lactose −, salicin + and the lactose +, salicin + strains may assume the rôle of opportunist pathogens, producing infections in various parts of the body and rarely may cause fatal septicemia. The source of such infections may usually be traced to the throat or genito-urinary tract. There is no evidence that these streptococci are of epidemic significance.

In cows, the lactose +, salicin + and the lactose +, salicin − strains are common causes of mastitis. The lactose −, salicin + strains have not been found in cows although they are capable of producing mastitis when inoculated into the udder.

In attempts to eradicate mastitis from dairy herds the human carrier of these streptococci should be considered.

The recognition of the double-zone hemolytic streptoocci supplies additional reason why diagnostic laboratories should no longer report all hemolytic streptococci as *Streptococcus hemolyticus*, a term which is inadequately informative and of doubtful validity as a specific name.

The author is grateful to those who have supplied cultures for this study, especially to Dr. Rebecca C. Lancefield, not only for many cultures but also for the serological grouping and typing of strains submitted to her.

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PHYSIOLOGICAL YOUTH AS AN IMPORTANT FACTOR IN ADAPTIVE ENZYME FORMATION

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It has been known for many years that bacteria, cultivated upon a specific substrate, may not ferment other materials unless cell multiplication occurs. Karström (1930) observed that cells grown in the presence of one sugar, then suspended in solutions of other sugars under conditions preventing multiplication, could not ferment the new sugars for long periods of time, if at all. Stephenson and Strickland (1933) showed that there was no natural selection in the case of formate adaptation by *Escherichia coli* and that the formation of the enzyme can occur while no cell division is taking place. Stephenson and Yudkin (1936) observed that top yeasts can adaptively ferment galactose without cell multiplication but no adaptation could be obtained with cultures incapable of growth. They state, however, that a study of their total and viable counts suggests that it is not the viable cells alone which are capable of adaptation. Stephenson and Gale (1937) found that the galactozymase of *Escherichia coli* was formed only when growth occurred in the presence of the specific substrate. This adaptation was proportional to the number of cells which had divided in the presence of the galactose.

The following experiments are the result of a search for the factors controlling the mechanism of adaptation. Old cells adapt themselves slowly if at all (Karström 1930) and young, growing cultures produce the new enzyme easily. Therefore, experiments were designed to test cells of various ages and to determine

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the stage of growth during which new enzymes are most easily manufactured.

METHODS

All of the experiments were performed with a culture of *Streptococcus lactis* (No. 125) which ferments all of the sugars characteristic of that organism when grown in nutrient media containing the various carbohydrates. The cells were grown in a medium consisting of 0.5 per cent peptone, 0.5 per cent tryptone, 1.5 per cent phosphate buffer at pH 7.0 and 0.5 per cent of the chosen carbohydrate (Rahn, Hegarty and Deuel, 1938). When the culture was at the desired age, the cells were centrifuged and resuspended in a solution containing 0.5 per cent peptone, 2.0 per cent phosphate buffer at pH 7.0 and 2.0 per cent of the carbohydrate to be tested. A heavy suspension of cells was used. With full-grown cultures, the cells from 250 cc. of medium were resuspended in 50 cc. of a 2 per cent buffer solution, and with growing cultures the ratio was changed accordingly. Frequent plate counts and Petroff-Hausser counts (Knaysi, 1935) showed no indications of growth in any of these suspensions. The rate of fermentation was determined by titration of 5.0 cc. samples with N/10 NaOH. If the cells were difficult to obtain, 1.0 cc. samples were titrated with N/18 NaOH, using a micro-burette.

Preliminary experiments showed that the enzymes attacking glucose, fructose and mannose were constitutive, i.e., produced under all circumstances, while the enzymes attacking all of the other sugars were adaptive, according to the terminology of Karström (1930).

A 12-hour old culture in glucose medium was centrifuged, and the cells resuspended in the buffer solution. One-third of this contained 2 per cent glucose, one-third 2 per cent sucrose, and one-third 2 per cent sucrose + 0.2 per cent glucose. Glucose was fermented from the start. No measurable acidity was produced from sucrose within 5 hours. The cells in sucrose + 0.2 per cent glucose produced 0.18 per cent lactic acid within the first hour, utilizing the glucose, but no further fermentation occurred until after 5 hours. The initiation of fermentation of one sugar had no effect upon acid production from other sugars.

A series of experiments was designed to determine the effect of the age of the culture upon adaptation. The cells were grown in glucose-tryptone broth, centrifuged out after various periods of incubation, and placed into buffered solutions of glucose and sucrose. Table 1 shows the gradual development of lactic acid from glucose and sucrose by cells centrifuged at various ages of the culture. All of the cells, regardless of age of the culture, fermented glucose from the start at rapid rates. The cells centrifuged when the culture was 2 hours old started fermentation in the sucrose medium earlier than cells obtained when the

TABLE 1

Per cent lactic acid formed from glucose and sucrose by glucose-grown cells of various ages

AGE OF CELLS	SUGAR	TIME IN HOURS					
		1	2	3	4	5	6
hours							
2	Glucose	.45	.50	.57	.57	.58	.58
2	Sucrose	.00	.05	.09	.14	.16	.19
12	Glucose	.23	.38	.41	.45	.47	.48
12	Sucrose	.00	.00	.00	.03	.03	.03
24	Glucose	.09	.22	.31	.43	.51	
24	Sucrose	.00	.00	.00	.03	.04	.04
48	Glucose	.04	.10	.14	.21	.26	
48	Sucrose	.00	.00	.00	.00	.01	.01

culture was older and adaptation required more time as the age of the culture increased.

A more extensive study was made of adaptation to galactose. 13.5 liters of glucose-tryptone broth were inoculated with 1.5 l. of a 36-hour culture of *Streptococcus lactis*. At the start and after every hour, samples of this culture were centrifuged and the cells resuspended in buffer plus glucose or galactose. The curves of these fermentations are shown in figure 1. Glucose was fermented from the start by cells of all ages. The number of cells per cubic centimeter of suspension could not be kept the same. The suspensions from the 3- and 4-hour old cultures contained about twice as many organisms per cubic centimeter as the 1- and 2-hour suspensions. The fermenting capacity per cell

per hour was 14.5 $\times$ 10^{-10} mgm. glucose for the 1 hour cells and had dropped to 9.3 $\times$ 10^{-10} mgm. when the culture was 4 hours old. These facts account for the differences in the rate of glucose fermentation.

Quite different are the fermentation curves for galactose. The cells collected when the culture was 1 hour old started fermentation soon after resuspension while the 4-hour cells required 9 hours for adaptation. Cells collected after longer periods of

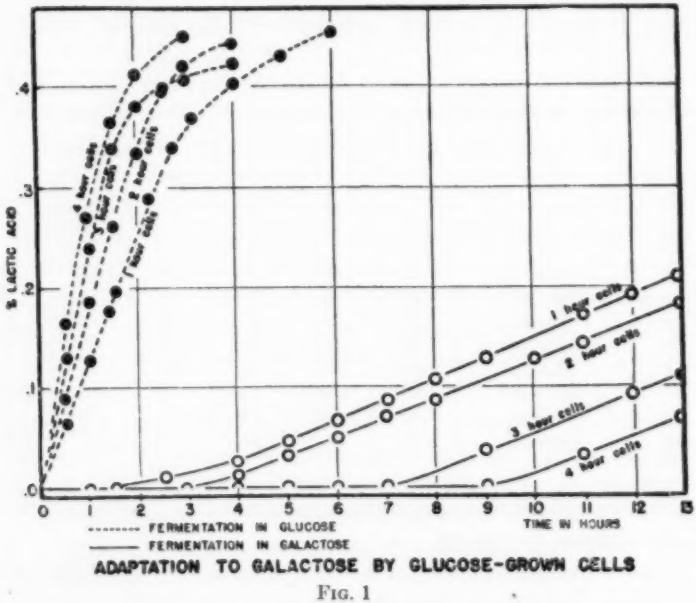

FIG. 1

incubation (not shown on the graph) did not produce any acid for 13 hours. The growth curve of the original culture supplying the cells for this experiment (fig. 2) showed that after 1 hour, the cells were just coming out of the lag phase. It is evident that as the culture passes from the stage of physiological youth into the logarithmic phase, adaptation becomes slower. Plate counts and Petroff-Hauser counts were made of all suspensions at frequent intervals and no increases in the numbers of cells could be detected.

Two other sets of experiments were performed in the same way, but with different sugars. In one, the cells were grown in glucose, and resuspended in buffer plus glucose, galactose, lactose, or sucrose. In the other experiment, the cells were grown in maltose, and tested in glucose, maltose, and sucrose. In general, the results were the same as those obtained before, as may be seen from table 2. Glucose-grown cells fermented glucose from the start. They showed rapid adaptation to galactose when

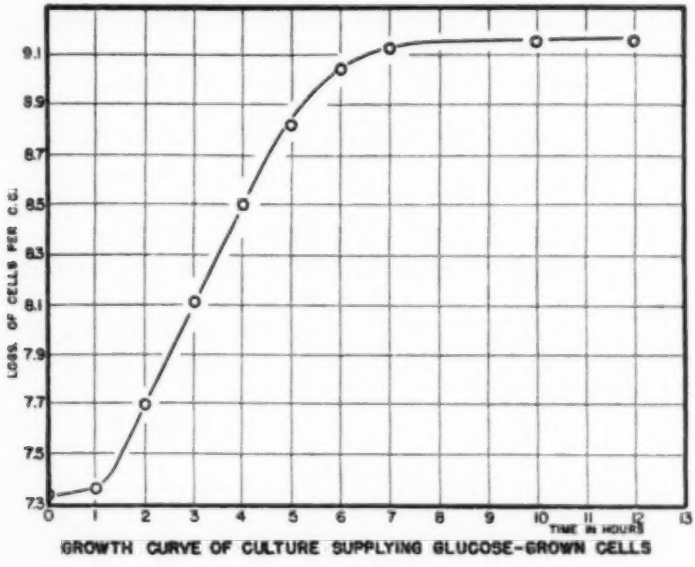

FIG. 2

obtained from a physiologically young culture, but required long initiation times when the culture was older. In lactose, the delay was 8 hours, with cells obtained from a physiologically young culture, and 20 hours, with cells from older cultures. The glucose-grown cells in sucrose showed no delay when the culture was just coming out of the lag phase. A growth curve showed that this culture had a 2-hour lag phase and therefore the cells centrifuged after 2 and 3 hours were physiologically young. The cells from older cultures exhibited a short delay.

In another experiment there was an adaptation time of 5.5 hours before glucose-grown cells produced acid from maltose.

The maltose-grown cells from cultures of all ages showed no delay in either glucose or maltose. This is not unexpected as the organisms had produced the adaptive maltase during growth in the original maltose culture, and the glucose enzyme is constitutive. Maltose-grown cells in sucrose required a short adaptation period when old, but showed no delay during physiological youth. It is interesting that maltose-grown cells from older cultures initiated fermentation in sucrose more rapidly than similar cells grown in glucose.

TABLE 2

Hours delay before adaptation occurs by cells of various ages grown in glucose and maltose

AGE OF CELLS	GROWN IN GLUCOSE				GROWN IN MALTOSE		
	Hours required for adaptation when placed in						
	Glucose	Galactose	Lactose	Sucrose	Glucose	Maltose	Sucrose
36	0	18	29	8	0	0	1.5
1	0	1.5	8	0.5	0	0	0
2	0	3	9	0	0	0	0
3	0	7	15	0	0	0	0
4	0	9	16	1.5	0	0	0
6	0	13	20	3	0	0	0.5
8	0	13	20	4	0	0	0.75
10	0	13	20	4	0	0	0.75

In every case where it was necessary for the organisms to adapt themselves to a new carbohydrate, the cells obtained from physiologically young cultures accomplished this much more rapidly than cells from a later stage of growth.

DISCUSSION

These experiments have shown that at the end of the lag phase, before the maximal growth rate or the logarithmic phase is reached, the cells are most adaptable to new types of food. During the logarithmic phase, while the rate of growth remains constant, adaptability decreases rapidly.

At this stage of early development, the cells are quite different from cells of slightly older cultures as was first shown by Sherman and Albus (1923) who termed them physiologically young. Later Bayne-Jones and Rhees (1928), Walker, Winslow, Huntington and Mooney (1934), Mooney and Winslow (1935) Huntington and Winslow (1937) and others have shown that the rate of metabolism per cell (production of heat, CO_2, NH_3, oxygen consumption, etc.) is maximal just before the logarithmic growth rate is reached.

Hershey and Bronfenbrenner (1938) state that "correlations of viable counts, centrifugeable nitrogen and turbidity, with oxygen consumption, indicate that the increased metabolism during the early portion of the growth period is quantitatively referable to increased average size of cell," which is not in complete agreement with the work of Huntington and Winslow (1937). While all these properties represent only quantitative changes, the case of adaptive enzymes is also a qualitative one, and therefore more significant, and in a class by itself.

It seems quite noteworthy that at the stage of rejuvenation of the old cells (Sherman and Albus, 1924), when the cell shows the most intensive metabolic activity, with distinct morphological changes (Henrici, 1928), the cell is also most ready for physiological changes and adaptations.

CONCLUSIONS

Cells from a mature culture of *Streptococcus lactis* in glucose-tryptone broth can not attack galactose, lactose, sucrose or maltose at once, unless they have been allowed to multiply in the presence of that sugar.

If cells are obtained from a glucose culture while still in the stage of physiological youth, they may attack these sugars either at once or with a short delay, even under conditions preventing proliferation.

The most rapid adaptation to new sugars is always observed with cells from cultures just coming out of the lag phase, during the period of physiological youth. During the logarithmic phase, adaptability decreases rapidly and continuously.

The ease of adaptation and the rate of loss of adaptability during the ageing of a culture varies greatly with each sugar.

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A METHOD FOR PRODUCING INCREASED CARBON DIOXIDE TENSION IN INDIVIDUAL CULTURE TUBES AND FLASKS

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During the past few years there has been a growing appreciation of the rôle of carbon dioxide in bacteriology. It has been demonstrated by a number of observers that the presence of carbon dioxide is apparently necessary for growth of all species of bacteria so far studied. Walker (1932) and Gladstone *et al.* (1935) suggest that the lag phase of the bacterial growth cycle is due to the failure of bacteria to multiply until a certain minimum amount of carbon dioxide is produced in the culture by their activities. If this is correct, it would appear that carbon dioxide should be supplied to cultures of feebly-growing organisms and to cultures in which the inoculum is small such as, for example, single cell cultures. Since the size of the inoculum, in terms of the number of bacterial cells, is not usually known when cultures are made from exudates, blood or other materials from suspected cases of disease, it follows that the routine provision of increased carbon dioxide tensions in such cultures would be desirable.

The use of increased carbon dioxide tensions has been recommended, especially for isolating gonococci, meningococci and *Brucella abortus*. A number of investigators, including McLeod and his associates (1934), Leahy and Carpenter (1936) and Thompson (1935) have demonstrated the value of incubating cultures for the isolation of gonococci from suspected cases in an atmosphere containing about 10 per cent CO_2. Cohen and Flem-

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ing (1918) as early as 1918 found the same percentage of CO_2 to be optimum for the isolation of meningococci from spinal fluids and nasopharyngeal cultures, a conclusion recently confirmed by Thompson (1935). The investigations of Huddleson (1921), Smith (1924), McAlpine and Slanetz (1928) and Wilson (1931) have shown that most strains of *B. abortus* grow best under a CO_2-tension of 5 to 10 per cent. The use of an increased tension of this gas in the primary isolation of bacteria of the *B. abortus* group has become routine in some laboratories in which appreciable numbers of cultures for the isolation of these bacteria are being made.

Increased carbon dioxide tension has found another important use in connection with the production of the exotoxins of some bacteria. Burnet (1930) introduced into common use the method of growing staphylococci in a semi-solid medium in an atmosphere containing about 20 per cent CO_2 in order to produce potent exotoxins. It has been found (Woolpert and Dack, 1933) also that staphylococcus enterotoxin, which is apparently distinct from the other exotoxins of staphylococci, can be produced more consistently in the presence of 20 to 30 per cent CO_2 than in the unaltered atmosphere. More recently enterotoxins have been obtained from streptococci, *Proteus*, *Salmonella aertrycke* and *Escherichia coli* by this method (Jordan and Burrows, 1935; Cary, Dack and Davison, 1938).

In spite of the demonstrated value of using increased carbon dioxide tensions in many phases of bacteriology, this procedure is not used routinely in most laboratories, even in cultures where its use is practically a requisite for success. This is due probably to the fact that it is cumbersome to use jars in which to incubate the cultures. Thompson's method (1935) of producing CO_2 in jars has simplified their use greatly. When culture jars are used, however, it is usually necessary to open the jars to see whether growth has occurred in the individual tubes, flasks or Petri dishes. If further incubation is necessary, the whole procedure of filling the jar with carbon dioxide must be repeated.

The method mentioned by Parker and Hudson (1926) for producing increased CO_2 tensions by burning a candle in the jar

in which the cultures are incubated is of distinct but limited value. CO_2 concentrations of about 3 per cent are apparently the maxima which can be produced by this technique (Nye and Lamb, 1936). This percentage is inadequate for the best results (McLeod *et al.*, 1934; Cohen and Fleming, 1918; McAlpine and Slanetz, 1928; Burnet, 1930). The use of the jar in this method is also subject to the criticism mentioned above.

Joyner and Jones (1937) have described a method recently by which the Spray (1930) anaerobic culture dish can be used for incubating individual cultures in an increased carbon dioxide tension. While this appears to be a valuable method, at least four criticisms may be levelled against it. Spray plates are relatively expensive. It is not possible, of course, to use liquid or semi-solid media in them which precludes their use for toxin production, for example. It is difficult to observe colonies on an opaque medium such as the "chocolate" agar commonly used in culturing gonococci without breaking the seal and remaking the whole culture. The most serious objection to the Spray plate method is that special culture media must be kept prepared in the plates even though cultures under CO_2 tension are only occasionally made in a laboratory.

The author has sought a simple method that can be applied to individual culture tubes and flasks, free from the objections mentioned above. It seems especially desirable to develop a method by which liquid media used for toxin production can be incubated in individual flasks. It also appeared that the method should allow the bacteriologist to utilize any type of medium desired for a particular purpose without making it necessary to place the medium in a special container beforehand.

Hall's (1937) modification of the method devised by Wright which is used routinely in this laboratory for absorbing oxygen from culture tubes was first tried. It was found, however, that, if the proper amount of bicarbonate to produce a certain CO_2 tension was placed on the cotton stopper, a great deal of the gas was lost before the rubber stopper could be inserted. In order to obviate this difficulty the following technique was evolved. The upper fluffy portion of the sterile cotton stopper of a test

tube or Florence flask is cut off and the remainder pushed as far down into the test tube or neck of the flask as possible without touching the medium. A short glass tube or shell vial of the proper size (about 10 x 35 to 40 mm. for the ordinary 16 to 18 x 150 mm. culture tube) is placed open end upward on the stopper. For flasks it is desirable to use larger vials, their size depending on the size of the flask. A gelatin capsule containing a measured amount of bicarbonate solution is placed in the inner tube or vial. A number 0 capsule has been found satisfactory for the test tube cultures but larger or smaller capsules may, of course, be used depending upon the amount of bicarbonate solution required for a particular purpose. A sufficient amount of sulphuric acid to cover the capsule, about 1 cc. for the test tube culture, is then placed in the vial and the whole culture tube sealed with a closely fitting rubber stopper. After about five minutes at room temperature the gelatin capsule begins to disintegrate and carbon dioxide begins to evolve gently. The method, which is illustrated by the diagram in figure 1, is much simpler than the description indicates and only a few seconds are required to make a culture.

Sulphuric acid with a bicarbonate is used as the source of carbon dioxide. Potassium bicarbonate is preferable to the sodium salt because of its greater solubility. To produce a 30 per cent CO_2 tension in a culture tube 16 to 18 x 150 mm., containing about 20 cc. of atmosphere above the medium, about 6 cc. of CO_2 are needed. At 20°C., assuming normal pressure, approximately 7.2 cc. of CO_2 are liberated from 0.1 cc. of three-molar potassium bicarbonate solution. For liberating the gas it is convenient to use an excess of acid so that it is unnecessary to measure it. Since it is desirable to provide sufficient acid to cover the gelatin capsule, we have found that 1 cc. of sulphuric acid diluted 1:30 as recommended by Thompson (1935) is suitable for a test tube culture. For a flask culture an appropriately larger amount of acid should be used.

Tests were made to be certain that the theoretical yield of CO_2 was liberated and diffused throughout the tube under the conditions described above. The atmosphere in the lower portion of

the culture tube, directly over the culture medium, was collected through a side arm and analyzed in a Yandell Henderson (Henderson and Greenberg, 1931) syringe gas analyzer, which has an accuracy sufficient for this purpose. When the pinchcock between the culture tube and gas analyzer was opened, the plunger in the syringe was forced out by almost exactly the number of cubic centimeters that should have been yielded theoretically

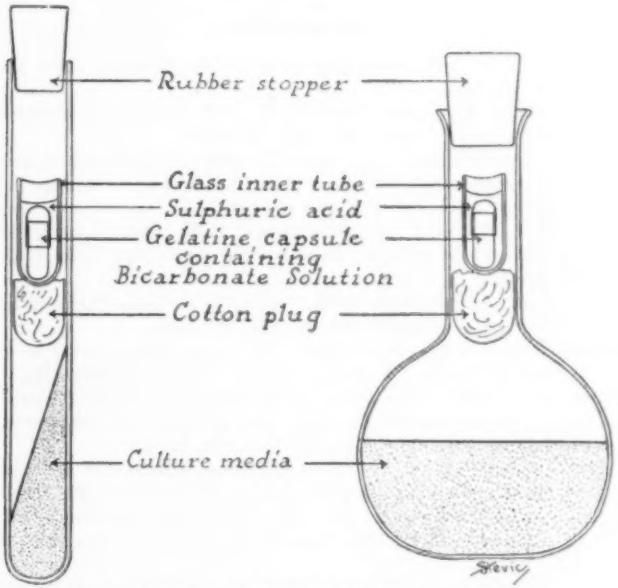

FIG. 1. SCHEMATIC DRAWING ILLUSTRATING METHOD

from the mixtures of bicarbonate and acid used. There were also very good approximations between the theoretical percentages of CO_2 which should have been present and the actual percentages found by gas analysis.

It has also been found that this method may be applied to anaerobic cultures. In this case, pyrogallic acid is placed in the inner vial, either directly or in a gelatin capsule, and 10 per cent sodium hydroxide is run in over it. The amounts should be

adjusted so that good absorption of oxygen takes place. About 1.5 grams of resublimed pyrogallic acid and 2.0 cc. of 10 per cent sodium hydroxide are satisfactory for the usual culture tube. An excess of pyrogallic acid should be maintained, of course, to prevent absorption of CO_2 by the alkali. In this connection the interesting possibility arises of using combined oxygen absorption and increased CO_2 tension to promote the growth of anaerobic or microaerophilic bacteria.

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GROWTH FACTOR REQUIREMENTS OF THE ROOT NODULE BACTERIA[1]

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It is known that yeast or plant extracts markedly stimulate the growth of the root nodule organism in media of purified ingredients. An essential growth factor or superior source of nitrogen has been invoked to explain the results, but neither theory has a broad experimental basis. The results of Allyn and Baldwin (1930) suggest that the extracts may affect favorably the oxidation-reduction potential of the medium. Allison, Hoover and Burk (1933) report that a specific co-enzyme for respiration of rhizobia can be extracted from commercial sucrose, azotobacter cultures and various plant or animal tissues. This extract markedly stimulates the growth of rhizobia in a medium of purified ingredients. Hoover and Allison (1935) state, "the growth of certain species, on the usual synthetic (sugar, inorganic salts, and nitrate) medium is negligible if pure ingredients are used, and that the addition of a small amount of growth factor is essential."

Thorne and Walker (1936) also found that an extract of azotobacter (or other substances) was able to stimulate growth of several species of rhizobia in a medium of highly purified materials but did not regard it as essential for growth. By substituting ammonium chloride or asparagin for potassium nitrate, they grew four species in repeated transfer for more than two months with no appreciable decrease in rate of growth of the organisms. Addition of reducing substances to Allison and

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Hoover's synthetic medium improved it, indicating that the potassium nitrate in this medium poised it at an oxidation-reduction potential too high for continued growth.

Clark (1936) was able to secure only very slight or no growth in the absence of added organic stimulants. Nilsson, Bjälfve and Burström (1938a) observed no growth in the synthetic medium of Allison and Hoover even if NH_4Cl or asparagine were used as the source of nitrogen. Likewise, the addition of reducing substances failed to favor growth; normal growth occurred only in the presence of yeast extract.

In view of the contradictory findings, it seemed desirable to investigate further the claims that certain unknown substances are necessary for growth of rhizobia, and to determine what factors are important for successful continuous transfer of the organisms in a synthetic medium.

EXPERIMENTAL

To avoid confusion in interpretation of results, the base medium adopted was that of Allison and Hoover (1934), consisting of mineral salts, one per cent mannitol, and 200 parts per million nitrogen as potassium nitrate. *Rhizobium trifolii*, Wisconsin strain 205 was used as the test organism.

The "coenzyme R" preparations employed were made from cultures of *Azotobacter vinelandii* according to Hoover and Allison's procedure (1935). In preliminary work a Petroff-Hauser direct count was made on fluid cultures of the organism to determine growth. Later, the growth response was measured by turbidity of fluid cultures as indicated by the Evelyn electrophotometer or development of giant colonies on agar.

Factors influencing rate of growth of R. trifolii in base medium

1. *Yeast extract and azotobacter extract.* A water extract of yeast (Fred, Baldwin and McCoy, 1932) and Allison's azotobacter extract were selected as representative of the numerous natural preparations known to stimulate the growth of *R. trifolii*. Tubes containing from 5 to 500 parts per million of these extracts were inoculated with a standard loop (approximately 500,000 cells per 10 ml. tube) from a 24-hour 10-per-cent yeast water

culture. The maximum stimulation occurred in the presence of 25 to 50 parts per million of either substance (table 1). It was noted that azotobacter extract consistently failed to replace, completely, yeast extract in fluid culture (fig. 1), while both were equally stimulative to growth on agar (Plate 1). In fluid culture, growth in the base medium was, in all cases, relatively slight, and failed completely after three or four transfers. From these data alone, one might conclude that certain stimulative organic enrichments are required for the prolonged growth of the organism in laboratory media. However, subsequent experiments demonstrate the need for modification of this opinion.

TABLE 1

Comparative stimulative effects of "coenzyme R" preparations and yeast extract
Counts in millions per ml.

MEDIA	2 DAYS	3 DAYS	4 DAYS
Coenzyme R, 5 ppm	92	206	285
Yeast extract, 5 ppm	160	385	586
Coenzyme R, 10 ppm	70	200	308
Yeast extract, 10 ppm	318	635	720
Coenzyme R, 25 ppm	75	280	341
Yeast extract, 25 ppm	485	856	840
Coenzyme R, 50 ppm	63	312	322
Yeast extract, 50 ppm	580	830	836
Control	<5	15	65

2. *Reducing substances.* Since the base medium was oxidizing in nature, the possibility of increased growth of the bacteria in the presence of reducing substances was investigated. Tubes containing sodium sulfite, sodium nitrite and sodium thioglycollate were inoculated from a twenty-four-hour culture as before. Table 2 indicates the amount of growth obtained in cultures supplied with optimal quantities of each compound. These substances all improved growth in fluid medium, but, as shown in Plate 1, they are without effect on agar. The use of mass inocula

apparently overcomes, within reasonable limits, effects of alteration in oxidation-reduction potential.

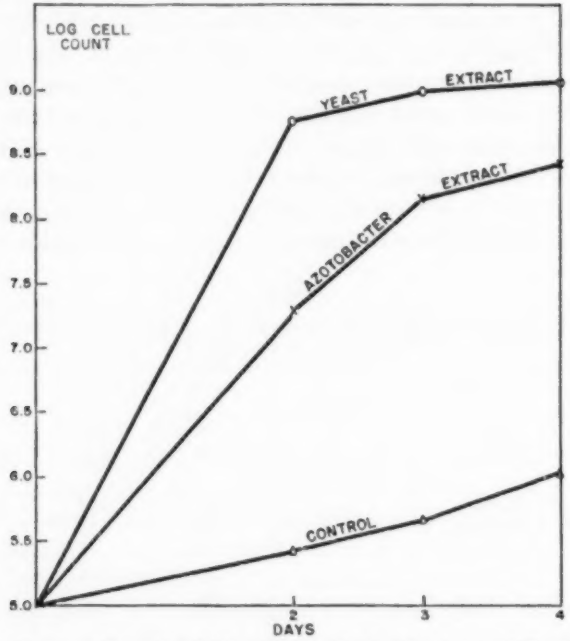

FIG. 1. Comparative stimulative effects of Azotobacter and Yeast extracts (50 ppm.) on growth of *Rhizobium trifolii* in fluid culture.

TABLE 2

Comparative stimulative effects of "coenzyme R" and reducing substances on growth of Rhizobium trifolii

Counts in millions per ml. Inoculum from 24-hr. culture

MEDIA	2 DAYS	3 DAYS	4 DAYS
Sodium nitrite, 25 ppm	72	245	318
Sodium sulfite, 25 ppm	75	188	345
Sodium thioglycollate, 10 ppm	85	390	416
Coenzyme R, 50 ppm	75	280	341
Control	<5	15	75

Figure 2 shows the relative stimulative abilities of thioglycollic acid and azotobacter extract, both at a concentration of 10 ppm.

At this concentration the stimulation of growth in fluid culture, due to azotobacter extracts, is of the same order as that effected by reducing substances. However, it is clear from Plate 1 that the former contains material of benefit to the organism for reasons other than its ability to alter the oxidation-reduction potential of the medium.

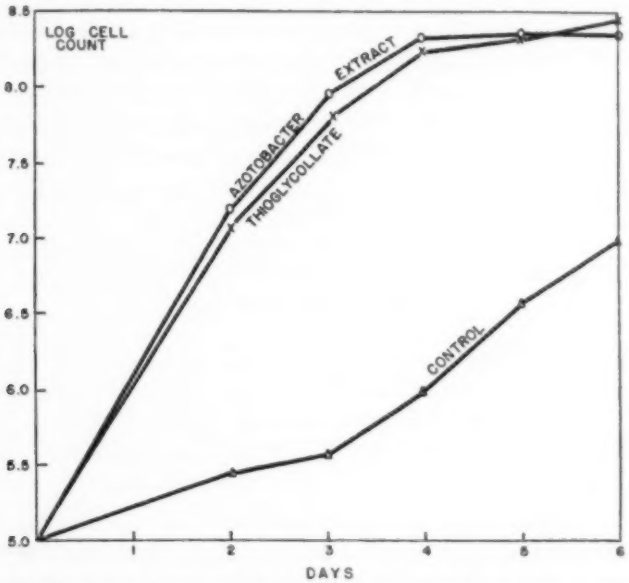

FIG. 2. Comparative stimulative effects of Azotobacter extract and thioglycollic acid (10 ppm.) on growth of *Rhizobium trifolii* in fluid culture.

Continuous growth of Rhizobium trifolii in synthetic media

Results of the foregoing experiments suggested the possibility of growing *R. trifolii* in fluid culture in the entire absence of unknown organic enrichments, over an indefinite period, providing the media were at a favorable oxidation-reduction potential. For the purpose of this experiment, the base medium enriched with 10 ppm. thioglycollic acid was selected. The original inoculation consisted of approximately 500,000 cells from a 24-hour 10-per-cent yeast water culture. Loop transfers were made every three days, and after thirty-four successive

transfers in this medium, the organisms were growing at the same rate as in the first transfer (table 3). After this number of transfers, it was concluded that any objection that growth could be due to activators from the original inoculum, was beyond consideration. Mannitol, which had been continuously extracted for 24 hours with absolute ethyl alcohol to remove any soluble activator, was used in a parallel experiment with identical results. Other reducing substances previously studied behaved similarly to thioglycollate. Purified glucose or sucrose could be substituted for mannitol with essentially the same results. Inoculation of a suitably reduced basal medium from either a yeast-extract enriched culture, or one grown in the absence of

TABLE 3

Growth of Rhizobium trifolii through thirty-four successive transfers in synthetic medium (base medium plus thioglycollic acid—10 ppm.)

Counts in millions per ml.

TRANSFER	2 DAYS	3 DAYS	4 DAYS
Original	86	295	382
1st transfer	64	170	284
2nd transfer	75	292	367
18th transfer	94	268	412
34th transfer	80	246	395

yeast extract for over thirty transfers, gave the same response. Evidently, then, the small amount of yeast extract carried in the inoculum from a 10-per-cent yeast-water culture does not influence the growth. In the presence of greater amounts of yeast extract, however, there is, as has been previously shown, a considerable stimulation beyond the growth induced by reducing substances.

It is concluded from these data, that *R. trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of the properly reduced base medium; and that although various tissue extracts are stimulative to growth, they are not required.

Growth factors synthesized by Rhizobium trifolii

Further study suggested that continuous transfer of *R. trifolii* in the base medium, properly reduced by thioglycollate, is dependent upon some highly active material, synthesized by the growing culture and transferred in sufficient amount with a loop inoculum (.005 ml.) to stimulate growth initiation in the new medium. Evidence for this view was the observation that cells removed from the medium in which they had grown, and washed free of their metabolic products, produced much less growth when inoculated into fresh media than did cells not washed. This separation of the organisms from the activators they produce is accomplished either by aseptic centrifugation of a fluid culture, or by suspending growth from the surface of an agar slope in fresh medium. An active culture filtrate, free from cells, can be obtained readily by ultra-filtration.

To determine the effect of culture products on the growth of *R. trifolii*, the following were used as inocula:

(a) A 48-hour culture growing in 10-per-cent yeast extract medium.
(b) A suspension of cells from a 10-per-cent yeast-extract agar slope in fresh 10 per cent yeast extract medium.
(c) A suspension of cells from a slope as in (b) in a sterile filtrate from culture (a).

All inocula were adjusted to contain the same number of cells. The results of this experiment (table 4) indicate that the bacteria during their growth synthesize some highly active material, important in growth initiation, which can be separated from the culture by Berkefeld filtration. That this effect is independent of the presence of yeast extract in the medium is shown in figure 3, which demonstrates rates of growth when the inocula used were:

(a) A growing culture, free of yeast or other extracts for 30 transfers.
(b) Cells from (a) centrifuged and resuspended in the fresh basal medium.

These examples, which are typical of a number of similar tests, illustrate the dependence of the organism on activators synthe-

TABLE 4

Influence of filtrate from growing culture on initiation of growth

Counts in millions per ml.

MEDIUM	INOCULUM	2 DAYS	3 DAYS	4 DAYS
Basal medium + 10 ppm. thioglycollate	*A*. 48-hour culture,	90	390	445
Basal medium + 15 ppm. thioglycollate	growing in 10 per	55	384	395
Basal medium + 20 ppm. thioglycollate	cent yeast ext. medium	72	341	380
Basal medium + 10 ppm. thioglycollate	*B* Suspension of	5	15	75
Basal medium + 15 ppm. thioglycollate	cells in sterile 10	5	5	83
Basal medium + 20 ppm. thioglycollate	per cent yeast ext. medium	5	5	35
Basal medium + 10 ppm. thioglycollate	*C*. Suspension of	106	390	428
Basal medium + 15 ppm. thioglycollate	cells in sterile	93	374	410
Basal medium + 20 ppm. thioglycollate	filtrate from *A*.	50	240	325

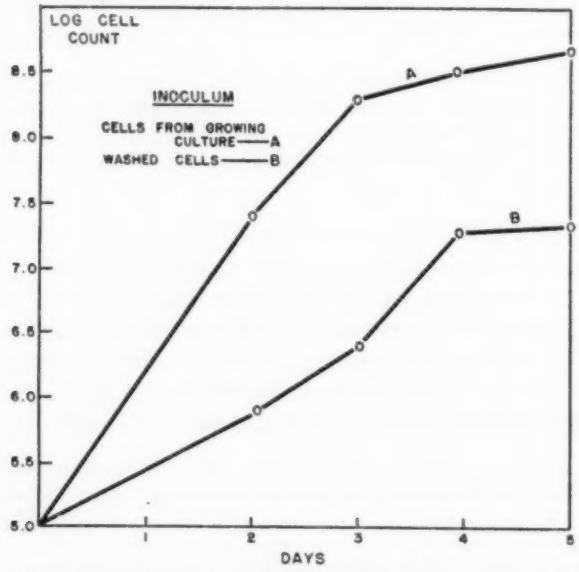

FIG. 3. Effect of type of inoculum on growth of *Rhizobium trifolii* in base medium plus thioglycollate.

sized during the period of active growth of the cells for growth initiation of small inocula. In the absence of this active material, growth is so slight that continued transfer is impossible.

Influence of heat on growth factor synthesized by R. trifolii

The heat stability of the stimulative factor liberated in growing cultures was determined by subjection of the Berkefeld filtrates obtained therefrom to a temperature of 100°C. Heating was carried out at neutrality and with the addition of 10 per cent N/1 sodium hydroxide or hydrochloric acid. After heating for periods of 30 minutes and one hour, the tubes were immediately cooled and adjusted to neutrality with sterile acid

TABLE 5

Influence of heat on stimulative properties of filtrates from growing cultures

Counts in millions per ml.

MEDIUM USED FOR SUSPENDING INOCULUM	2 DAYS	3 DAYS	4 DAYS
Filtrate untreated	105	340	385
Filtrate heated at pH 6.8, 30 min.	<5	20	74
Filtrate heated at pH 6.8, 60 min.	<5	17	65
Filtrate heated with N/10 NaOH, 30 min.	<5	32	86
Filtrate heated with N/10 NaOH, 60 min.	7	23	51
Filtrate heated with N/10 HCl, 30 min.	18	25	72
Filtrate heated with N/10 HCl, 60 min.	7	30	90
Control	<5	25	84

or alkali. One drop of a heavy suspension of cells was added to each differently treated filtrate, so that an inoculum of one loop taken from it carried approximately 200,000 cells. Results of this experiment showed that destruction of the growth-stimulating properties of the filtrate had occurred in every case (table 5). This is in marked contrast to the heat stability of the stimulative ingredients of yeast or azotobacter extract.

Activity of growth factor synthesized by R. trifolii

A quantitative study of the activity of *Rhizobium* culture filtrates in stimulating growth of the same organism was under-

taken, using a modified method for growth measurement. In this and subsequent experiments, twelve-ounce bottle plates were sown with giant colonies (see Plate 3). Usually, 11 or 12 colonies were sown to insure obtaining growth of at least 10. After 72 hours' incubation at 28°C., growth from ten colonies was removed and suspended in 10 ml. distilled water, and the turbidity read in an Evelyn electrophotometer. From a standardization curve the electrophotometric readings could be converted directly into numbers of cells. Parallel experiments were run, using fluid cultures as before, with the exception that growth was determined in the electrophotometer after 48 hours, instead of by direct count.

It was found more convenient in these studies to employ autolyzed cultures of the organism (8 to 10 weeks old) as a source of the growth factor. After the cells had settled, the supernatant medium was removed and, by pasteurization at 80°C. for 5 minutes, was rendered sterile without any destruction of stimulative activity. These culture autolysates were prepared from cultures grown in purely synthetic media for over ten transfers.

To determine the potency of the culture autolysate as a stimulant for *R. trifolii* it was tested at concentrations of .0001, .001, .01, .1 and 1.0 ml. per 10 ml. of medium. Additions were made both before and after autoclaving, to compare the effects of heated and unheated autolysate at various concentrations. At the same time, an autolysate of *Azotobacter chroococcum* prepared in the same manner, was tested to determine whether or not this organism also synthesized the factor.

The results (fig. 4) indicated that the *Rhizobium* autolysate was most active when added to the medium in concentrations between .001 and .01 ml. per 10 ml. of medium. The optimum concentration of autolysate (which is approximately equal to the amount of medium carried from an old to a new culture in a loop inoculation) is the same whether determined by plate or fluid cultures. Of interest, is the observation that additions beyond 0.1 per cent resulted in loss of stimulative action; this is

a peculiarity which assisted in identifying the active material. Autoclaving inactivated the autolysate at all concentrations.

Azotobacter autolysate apparently contains very little, if any, of the heat-labile *Rhizobium* factor, but does cause response at higher concentrations due to the presence of a heat stable substance, presumably that described by Allison and Hoover.

Although *Azobacter* did not synthesize appreciable quantities of the *Rhizobium* factor, it was thought possible that a more

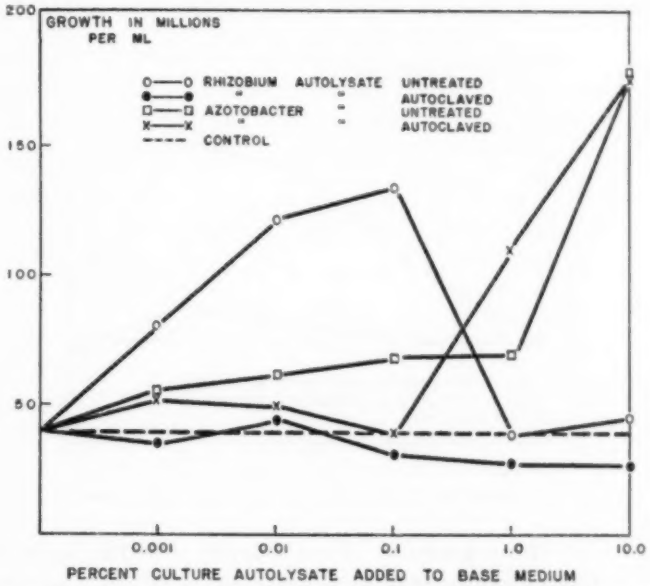

FIG. 4. Effect of heat on growth stimulants synthesized by *Rhizobium trifolii* and *Azotobacter chroococcum* determined by the giant colony method.

closely related species such as *Phytomonas tumefaciens* might do so, unless the phenomenon were strictly confined to the nodule organisms. The effects of *Phytomonas* autolysate on the growth of *R. trifolii* as determined by the giant colony technic are shown in figure 5. It appears that *Phytomonas tumefaciens* synthesizes the stimulative material in approximately the same quantity as do the rhizobia themselves.

Thiamin and riboflavin as growth factors for R. trifolii

From certain of the properties already discussed, it appeared possible that the growth activator synthesized by the rhizobia might be some known constituent of the vitamin B group, such as thiamin or riboflavin (or the unstable complexes which these substances form in the living cell). In order to test this hypothesis, inocula for both giant colony and fluid cultures were employed consisting of washed cells, which permitted detection of the factor or factors which could replace the material syn-

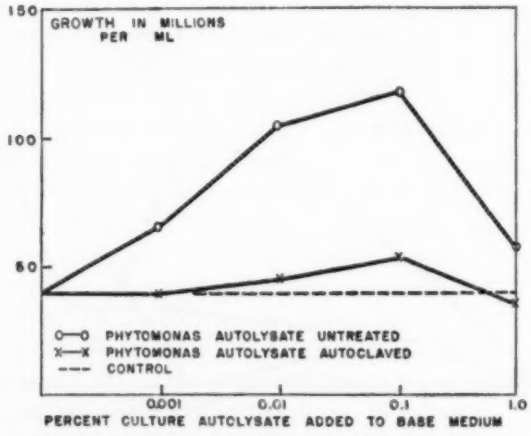

FIG. 5. Synthesis of *Rhizobium* factor by *Phytomonas tumefaciens*

thesized by the organisms. Pure thiamin and riboflavin were used in these experiments.

In preliminary studies, both of these substances were found to possess marked activity, but the organisms exhibited great sensitivity to only slight alterations in the concentration of the activator. The activity of these vitamins appeared to be limited to a narrow range of concentrations around 0.1 microgram per ml. This range of activity was studied more closely with the results, for thiamin, shown in Table 6. A similar experiment with both thiamin and flavin is shown in figure 6. In all cases it will be observed that as the concentration of either vitamin

increases beyond the optimum its stimulatory effect decreases. This is of interest in view of the fact that the factor or factors synthesized by the rhizobia possess the same peculiarity. This effect is shown clearly in the accompanying Plates 2 and 3.

If thiamin and flavin are the actual substances synthesized by *R. trifolii*, it would be expected that they would not give further stimulation of growth if the inoculum were taken direct from a growing culture. This was found to be the case (see table 6). In the presence of sufficient of the activator, further additions

TABLE 6

Influence of inoculum on response of Rhizobium trifolii to vitamin B_1 (thiamin)

Counts (millions per ml.) on 48-hour fluid cultures calculated from electrophotometer turbidity measurements

MEDIA, VITAMIN B_1	INOCULUM	
	Washed cells	Growing culture
micrograms / ml.		
0.2	65	161
0.18	81	149
0.16	140	114
0.14	141	127
0.12	150	123
0.10	155	110
0.08	147	116
0.06	106	116
0.04	64	155
0.02	50	147
0.00 (control)	43	145

had either very little stimulative influence, or actually depressed growth. The fact that vitamin B_1 has very little effect when inocula are from actively growing fluid cultures has already been reported by Laird and West (1938).

From these and other experiments which gave similar results, it was concluded that either thiamin or flavin alone can replace, at least in part, the culture autolysate, thus enabling washed cell suspensions to grow nearly as readily in the reduced medium as cells inoculated directly from an actively growing culture.

Synthesis of vitamin B_1 and flavin by R. trifolii

Since thiamin and flavin were strongly suspected of being identical with the *Rhizobium* factor, it became necessary to determine the ability of the organism to synthesize these vitamins. For this purpose, a very sensitive quantitative method for assaying vitamin B_1 was developed, based on Knight's finding (1937) that in appropriate media, the growth of *Staphylococcus aureus* is proportional to the amount of vitamin B_1 present.

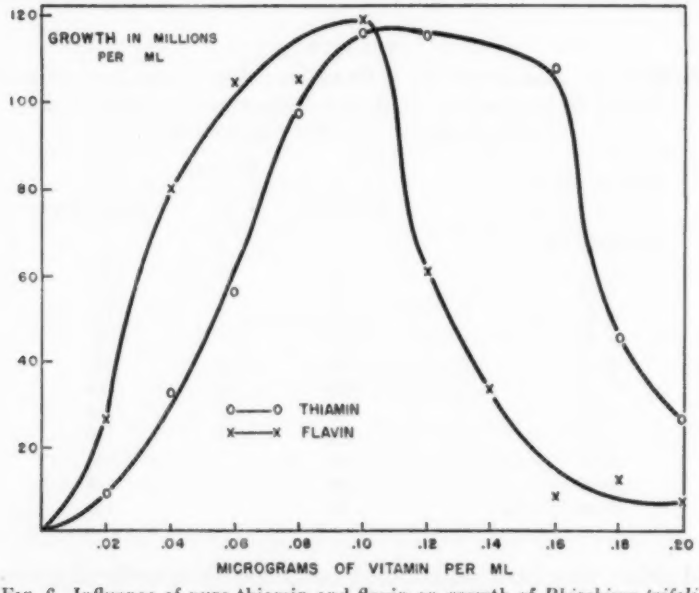

FIG. 6. Influence of pure thiamin and flavin on growth of *Rhizobium trifolii*

Details of this procedure are reported elsewhere (West and Wilson, 1938). Results of analyses of *R. trifolii* cultures grown on a synthetic, vitamin-free medium indicated an average of 19.6 micrograms of vitamin B_1 per gram of dry cells. This relatively high value indicates that the vitamin B_1 content of *R. trifolii* cells closely approximate that of yeasts.

The flavin content of *R. trifolii* was estimated by the method developed by Snell (unpublished data) involving growth re-

sponse and acid production by *Lactobacillus casei*. An average from four separate determinations showed the flavin content of *Rhizobium* cells to be 0.370 microgram per milligram dry cells. These cells are therefore rich in flavin, since the *Clostridium butyricum*, reported by Warburg and Christian (1933) to be high in flavin, contained only .09 microgram per milligram.

Since *R. trifolii* synthesizes thiamin and flavin in such appreciable amounts, it would appear that these substances must be of great importance in the metabolism of the organism. Possibly, one of the reasons for the existence of the lag phase in *Rhizobium* cultures is the necessity for synthesis of sufficient of these stimulants before multiplication can occur. Therefore, if the inoculum be washed, and the cells deprived of most of these growth factors which would ordinarily be carried from the previous culture, the lag is prolonged, in many cases indefinitely. The addition of vitamin B_1, flavin or culture filtrate overcomes this inactivity.

Nature of the growth factor synthesized by Rhizobium trifolii

From a comparison of the thiamin and flavin contents of the autolysates from *Rhizobium trifolii* cultures and the amounts of these vitamins which must be added in pure form to produce stimulation, it was found that more of either vitamin is required for growth response when added singly than occurs in the bacterial autolysates. There are two possibilities to account for this apparent discrepancy: (*a*) thiamin and flavin in combination may have a greater than additive effect; (*b*) these vitamins may exist as protein or other complexes with greater activity than they possess in the free state. Evidence has been obtained that both of these possibilities may be involved.

Experiments on the combined effects of thiamin and flavin showed that if either vitamin is present in optimum concentration, addition of the other reduces activity. However, if both are present in amounts too small to be effective alone, the combination is stimulative. This interaction of flavin and thiamin is shown in figure 7.

It would seem that thiamin is present in the cell as an unstable

complex since, in assaying autolysates from *Rhizobium* culture for vitamin B_1, it was necessary to heat for 5 minutes (N/10 hydrochloric acid) at 100°C., a treatment liberating the free vitamin but not destroying it. On assaying the unheated autolysate, only 0.3 microgram of vitamin B_1, per gram of dry cells was found, whereas after a short heating the vitamin B_1, was freed (19.6 micrograms per gram).[2] In assaying for flavin it was likewise necessary first to liberate the free substance by heat treatment.

In combined form the vitamins are apparently more stimulative since, following the splitting of these complexes by heat, a

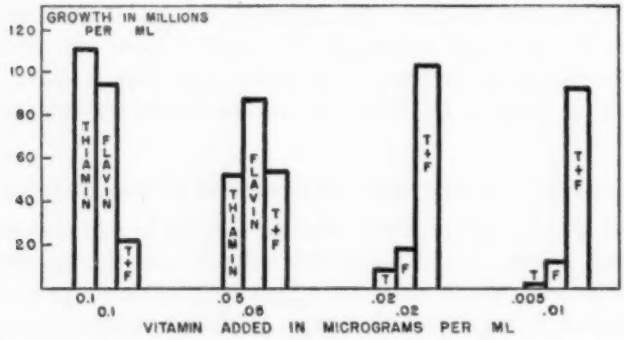

FIG. 7. Effect of combinations of thiamin and flavin on *Rhizobium trifolii* as determined by growth in fluid culture.

treatment which is sufficient to liberate the free vitamin but insufficient to destroy it, the activity of the *Rhizobium* autolysate is largely lost.

For these reasons it can be well understood that either flavin or thiamin alone does not possess the same degree of activity as the culture autolysate which may contain both of these vitamins in correct combination and in more suitable form.

[2] Recent work by Sinclair (1938) indicates that the vitamin B_1 of blood is also in combined form (not co-carboxylase). In assaying B_1 of blood by Meikeljohn's method it is necessary to subject it to a short heating capable of liberating the vitamin but insufficient to cause any destruction of the vitamin itself.

DISCUSSION

The more important results of this investigation may be summarized as follows:

1. Heat- and alkali-labile substances are synthesized by *R. trifolii* itself, which, when present in sufficient amount in the inoculum, permit considerable growth of the organism in a medium of purified ingredients. Even small inocula (200,000 to 500,000 per 10 ml.), washed free of the metabolic products of the surrounding medium, initiate growth satisfactorily if they are suspended in a filtrate from a growing culture. In fluid culture, the total population in this case may reach as high as 250–400 million per ml. in 72 hours. If the culture filtrate is merely added to the synthetic medium, the total population reaches only 100–150 million organisms per ml. in 72 hours. These essential factors occurring in the filtrate from an actively growing culture on synthetic medium can be replaced, at least partially, by the direct addition of riboflavin and thiamin in suitable concentration to the synthetic medium. Washed cells in the presence of the vitamin-enriched medium reach a density of 75 to 150 millions per ml. in 3 days. In the absence of culture filtrate, thiamin, or riboflavin, little or no growth occurs with such an inoculum.

The factor essential in growth initiation of *R. trifolii*, which is synthesized by actively growing cultures of the same organism has been termed the *Rhizobium* factor in order to distinguish it from the heat-stable substance of Allison and Hoover. The *Rhizobium* factor presumably consists of thiamin and riboflavin and possibly some other unidentified compounds, all of which the organism is able to synthesize, *once growth has been initiated.*

The use of the term "essential" as applied to the *Rhizobium* factor may appear to be somewhat arbitrary. It is used in the sense that the factor (or factors) must be present in order that growth may be initiated in a properly poised synthetic medium. Ordinarily, sufficient is carried over in a loop inoculum directly from an actively growing culture, and in this case the essential factor is furnished by the organisms themselves. If the organism is separated from its previously synthesized growth factor, it must be added to the medium, else little or no growth occurs.

But for initiation of growth in the synthetic medium the "essential" factor must be present, whether supplied directly in preparation of the medium or indirectly in the inoculum. In either case, once growth is initiated, the organism can synthesize sufficient of the factor to enable it to be transferred continuously even though maximum populations are not reached.

2. A second factor described by Allison and Hoover is also concerned with the nutrition of *R. trifolii*. This factor, termed "Coenzyme R," stimulates the growth of these bacteria, but does not appear to be essential for their continuous transfer. In the presence of optimum quantities of the factor of Allison and Hoover, the total population may reach 300–750 millions of organisms per ml. in 3 days. "Coenzyme R" differs markedly from the *Rhizobium* factor, and the two can be readily distinguished on the basis of stability and physiological effects.

It is possible that the heat-stable and heat-labile factors may be related as, for example, the factor described by Allison and Hoover may provide an organic nucleus which the organism converts readily into its necessary growth factors. According to Allison and Hoover "Coenzyme R" is concerned with respiration; the established importance of both thiamin and riboflavin to respiratory processes might be used as supporting this suggestion of relationship. However, much more experimentation will be required to investigate this phase of the subject.

3. In the light of these studies on the importance of condition of inoculum in determining whether or not growth will occur in a synthetic medium, it is believed that many of the discrepancies occurring in the literature on this point can be explained. For example, Thorne and Walker were able to obtain continuous transfer in synthetic medium starting with an inoculum from a yeast-extract slant and subsequently transferring one ml. of the 4 day old culture to 25 ml. of fresh liquid medium. Objection to their work on the grounds that they were transferring "Coenzyme R" from the original yeast extract culture can hardly be sustained, since they carried the organisms through 10 to 20 transfers. Under even more rigorous conditions with respect to size of inoculum, we have maintained continuous culture for over 30 transfers with no evidence of loss in reproductive power.

Nilsson, *et al.* (1938a), on the other hand, have recently reported complete inability to obtain growth on a synthetic medium even in the presence of reducing substances; they attribute the success of other workers to faulty technique. Although they do not give much detail regarding the preparation of their inoculum, they do state that they used few cells, obtained by dilution of a culture. This probably corresponds to our inoculum of cells suspended in fresh medium and therefore deficient in *Rhizobium* factor. We are in complete agreement with them that under such conditions no growth results even on inoculation into an adequately reduced medium. And it is also understandable that under such conditions they find vitamin B_1 stimulative (Nilsson, Bjälfve and Burström (1938b), since it is only when an inoculum deficient in the *Rhizobium* factor is employed, that vitamin B_1 induces any marked response.

SUMMARY

1. *Rhizobium trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of a properly reduced carbohydrate mineral-salts medium.

2. Although various tissue and microbial extracts contain a heat-stable substance (or substances) stimulative to growth, these are not required for successful continued transfer of the organism in a synthetic medium.

3. Continuous transfer of *Rhizobium trifolii* in a synthetic medium is dependent on a factor synthesized by the growing culture which is ordinarily transferred in sufficient amount in the inoculum to stimulate growth initiation.

4. This factor can be separated from cultures of *Rhizobium* by ultrafiltration. It is readily destroyed by heat.

5. Small inocula, washed free of metabolic products of the culture from which they were taken, produce little or no growth in the absence of the *Rhizobium* factor.

6. In the presence of certain specific amounts of thiamin or flavin, either vitamin alone is capable of replacing to some extent the stimulative material synthesized by *R. trifolii*. However, certain combinations of both vitamins are more active than either one used singly.

7. *Rhizobium trifolii* synthesizes appreciable amounts of both vitamin B_1 and riboflavin. These vitamins are present in culture autolysates or filtrates as complexes which are readily destroyed by heat, resulting in decreased activity.

8. Further evidence is presented which indicates that the activity of the metabolic products of *R. trifolii*, in stimulating growth initiation of the same organism, is due, at least in part, to the presence of thiamin and flavin in those products.

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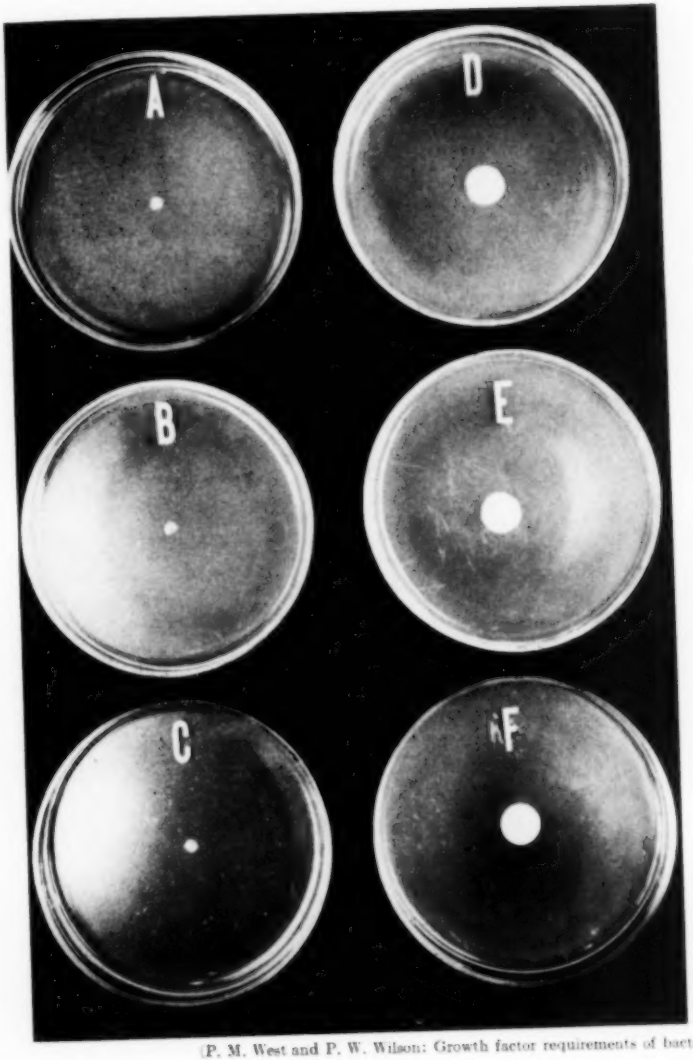
PLATE 1

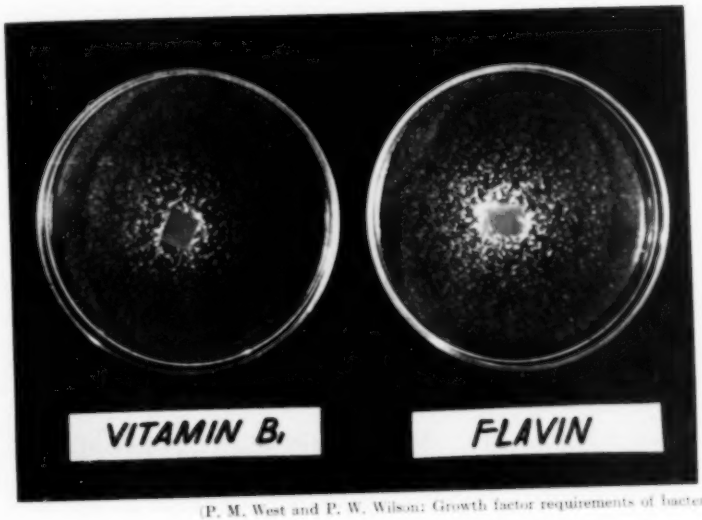
Influence of various supplements (25 parts per million) on growth of giant colonies of *Rhizobium trifolii*. A: Control. B: Casein hydrolysate. C: Thioglycollic acid. D and E: Allison's Azotobacter extract. F: Yeast extract.



(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)

PLATE 2

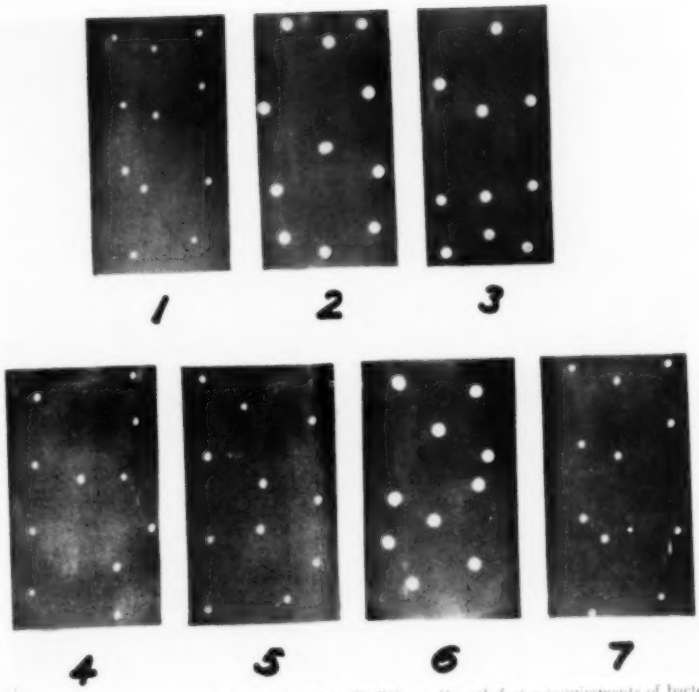
Stimulation of growth of *Rhizobium trifolii* in base medium around blocks containing 0.2 microgram per ml. vitamin B_1 and flavin.



(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)

PLATE 3

Effect of varying concentration of vitamins on growth of *Rhizobium trifolii*. 1 and 4: Controls. 2 and 3: 0.1 and 0.2 microgram vitamin B_1 per ml. 5, 6, and 7: 0.05, 0.1 and 0.5 microgram flavin per ml.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THE DETERMINATION OF HISTAMINE IN BACTERIAL CULTURES

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The production of histamine in bacterial cultures has been the subject of a number of investigations. According to these reports, the power to produce this amine is confined to certain strains of only a few species of bacteria: *Escherichia coli* and related organisms (Mellanby and Twort, 1912–13; Koessler and Hanke, 1919c; Hirai, 1933); *Salmonella enteriditis*, *S. schottmuelleri*, and *S. morgani* (Koessler, Hanke, and Sheppard, 1928); a variety of *Aerobacter aerogenes* designated as *B. aminophilus-intestinalis* (Bertrand and Berthelot, 1913; Jones, 1918); and *Clostridium welchii* (Kendall and Schmitt, 1926; Kendall and Gebauer, 1930).

Several methods have been used to identify histamine in cultures. Ackermann (1910), Bertrand and Berthelot (1913), Mellanby and Twort (1912–13), Hirai (1933), and Kendall and Gebauer (1930), prepared the crystalline di-picrate of the base. This method, while reliable when positive, is qualitative only, because of the large losses in purification. The process is laborious, and rather large quantities of culture must be worked up.

Mellanby and Twort (1912–13) and Jones (1918) also determined histamine in cultures by the Dale and Laidlaw (1910–11) technic, employing the isolated guinea pig uterus. Kendall and Schmitt (1926) used guinea pig intestine in a similar technic. These, as well as other physiological methods, are open to the objection that substances other than histamine may provoke the same response. As Best and McHenry (1931) point out, no one single physiological method for histamine assay is reliable; if the

physiological method is to be used, only a complete biological analysis can establish the presence of histamine.

Koessler and Hanke (1919a) determined histamine colorimetrically by the Pauly diazo reaction. As many other substances react with the diazo reagent, it was first necessary for them to isolate the histamine in a fairly pure form (Koessler and Hanke, 1919b). This was done by extracting the cultures with amyl alcohol after making them strongly alkaline. The histamine was recovered from the amyl alcohol by shaking with dilute acid. It was then determined quantitatively by means of the diazo reaction, for which they described an improved technic.

In our investigation of histamine production by bacteria, we first made use of the extraction method of Koessler and Hanke (1919b). It was soon found that this method presented certain difficulties. It was very time-consuming. It required large amounts of reagents, some of which are expensive. The colors obtained with the extracts often do not match well with the standard, especially if glucose is present in the culture medium. We therefore changed the extraction method to overcome these drawbacks.

As extracting solvent, we used a mixture of three parts of chloroform to one part of amyl alcohol. "Reagent" grades of these solvents were used; after being mixed, they were shaken with about one-fifth of their volume of 1 per cent sulfuric acid; then with several changes of distilled water. This removes impurities that would be extracted later with the histamine.

This mixture of chloroform and amyl alcohol is a more selective solvent for histamine than is amyl alcohol alone. Its use avoids the extraction of the interfering substance produced from glucose, as well as some other interfering substances.

For the extraction, we used the excellent method devised by Widmark (1926), by which quantitative extractions of small amounts of material can be made with a minimum of labor and attention. Widmark's apparatus consists of two small connected chambers; in one of these is the solution to be extracted; in the other is placed a solution in which the extracted substance is

changed to a form insoluble in the extracting solvent. A suitable immiscible organic solvent connects the two chambers; when filled, the apparatus is tilted continually, so that the extracting solvent flows back and forth between the two solutions. The apparatus described by Widmark was intended for solvents lighter than water; as the chloroform-amyl alcohol mixture is heavier than water, we devised a different form of apparatus. This consists of two glass bulbs connected by a vertical U-tube

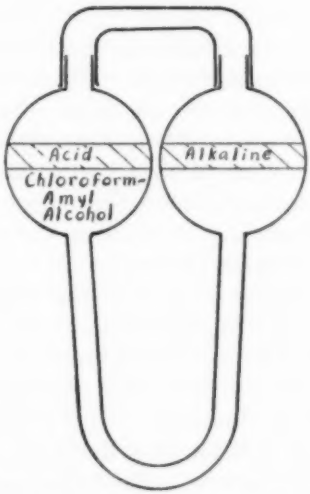

FIG. 1. DOUBLE BULB EXTRACTION APPARATUS

(fig. 1). The bulbs have a diameter of 4.5 to 5.0 cm., a size that is suitable for extracting 10 cc. of fluid. The removable tube connecting the two orifices was added to prevent loss of chloroform by evaporation.

The tilting apparatus consists of a rack with slots to hold 12 such "double bulbs." The rack is pivoted in the middle; the tilting is done by a motor with a reducing gear, which tilts the rack 8 times per minute through an angle of 8 degrees on each side of the horizontal.

By our method, four main steps are involved in the determination of histamine in bacterial cultures:

I. The preparation of the acid extract.
II. The removal of volatile bases and amyl alcohol.
III. The quantitative determination of histamine by means of the diazo reaction.
IV. The preparation of histamine di-picrate from the acid extract (not done in every case.)

I. THE PREPARATION OF THE ACID EXTRACT

About 30 cc. of the washed chloroform-amyl alcohol mixture were introduced into a double bulb. Then, 10 cc. of 0.5 per cent sulfuric acid were pipetted into one bulb. Ten cubic centimeters of the test fluid (bacterial culture) were neutralized, then made alkaline by adding 1.5 cc. of 15 per cent Na_2CO_3 solution, and introduced into the other bulb. (Stronger alkali must not be used, otherwise interfering substances are extracted and the histamine readings become too low.)

The connecting bridge over the two orifices was then put into place, and the double bulb set in a slot in the tilting rack. The apparatus was then run for 24 hours at room temperature.

As the solution to be extracted is strongly alkaline, the histamine is present as the free base. This is slightly soluble in the extracting fluid; and a little of it dissolves in the chloroform-amyl alcohol at the interface. The tilting moves the extracted histamine to the other bulb, where it comes in contact with the dilute sulfuric acid, forming the sulfate. This is no longer soluble in the extracting fluid; it enters the aqueous layer, from which it cannot return. This process continues until all of the histamine is in the acid extract. Other bases, such as ammonia and the volatile amines, will also be transported, but the great bulk of impurities will be left behind. Not the slightest trace of such substances as histidine, peptone, etc., ever appears in the acid extract. The presence of proteins in no wise interferes. The chloroform amyl-alcohol layer often becomes very turbid during the extraction; this turbidity has no significance and can be disregarded.

Sulfuric acid is the acid of choice in this extraction, as none of it will be transported to the alkaline side. Organic acids that are soluble in the choloroform amyl-alcohol will be rapidly carried over. Hydrochloric acid is transported slowly; it can be used in this extraction if allowance is made for the fact that some of it will pass over to the alkaline bulb.

Factors that influence the speed of extraction:

The first factor that influences the speed with which histamine will be extracted is the composition of the extracting fluid. Pure chloroform, in this apparatus, will extract histamine, but the rate is too slow to be of use. The addition of as little as 5 per cent of amyl alcohol greatly increases the speed of extraction. Our experience indicates that 25 per cent of amyl alcohol is most practical. With still more amyl alcohol, the speed of extraction is increased, but the specific gravity of the mixture becomes dangerously low; more interfering impurities will also be extracted.

Either the normal or the iso-amyl alcohol can be used; we found no difference. Butyl alcohol may be substituted for the amyl alcohol. Even ethyl or methyl alcohol can be utilized; with these lower alcohols, much of the alcohol will be in the aqueous layers. Instead of chloroform, one may use tetra-chlor-ethane or carbon tetrachloride. In our hands, however, chloroform and amyl alcohol gave the most satisfactory results.

Another important factor that determines the speed of extraction is the ratio of the area of the extracting surface to the volume of fluid to be extracted. This ratio should be high; in other words, the fluid to be extracted must form a *shallow* layer. In our apparatus, this layer was less than 1 cm. deep.

Other factors that influence the rate of extraction are the speed of tilting, the angle of tilt, and the internal diameter of the connecting U-tube. A large quantity of the extracting solvent must stream from one bulb to the other at each tilt of the rack. If the rate of tilting is too rapid, or if the angle of tilt is insufficient, or if the connecting U-tube is too narrow, then only small quantities of solvent will surge back and forth and the

rate of extraction is diminished. With our apparatus, 8 to 15 tilts per minute were found optimal.

We determined the rate of extraction of histamine in two different ways. By the first method, pure solutions of histamine di-hydrochloride were extracted for different periods and both the acid extract and the alkaline residue were assayed. By

TABLE 1

Rate of extraction of histamine from a solution containing 0.730 mgm. of histamine di-hydrochloride in 10 cc.

	FOUND IN ACID EXTRACT		FOUND IN ALKALINE RESIDUE	IN CHLOROFORM-AMYL ALCOHOL (BY DIFFERENCE)
	mgm.	*per cent*	*mgm.*	*mgm.*
2-hour extraction	0.370	50.7	0.240	0.120
4-hour extraction	0.490	67.1	0.150	0.090
8-hour extraction	0.690	94.5	0.020	0.020
18-hour extraction	0.730	100.0	0	0

TABLE 2

Rate of extraction of histamine from a culture of Salmonella schottmuelleri on meat extract-peptone-histidine-glucose medium

	FOUND IN ACID EXTRACT (10 cc.)		REMAINING IN ALKALINE RESIDUE* (10 cc.)
	mgm.	*per cent*	*mgm.*
2-hour extraction	0.640	28.8	1.580
4-hour extraction	1.050	47.7	1.150
8-hour extraction	1.610	73.9	0.570
12-hour extraction	1.870	83.9	0.360
24-hour extraction	2.13	98.1	0.040

* Determined by re-extracting the alkaline residue for 36 hours.

the second method, a culture of a histamine-producing bacterium was extracted for different time intervals; the acid extracts were removed and the alkaline residues were extracted again for 36 hours. The results of such experiments are shown in tables 1 and 2.

Table 1 shows that histamine is very rapidly extracted from solutions of pure histamine; in 8 hours, 94.5 per cent was removed,

and within 18 hours, all of it. Extraction of cultures, however, takes place more slowly. In 8 hours, only 73.9 per cent of what was probably the total histamine was extracted; even after 24 hours, a slight trace was left. According to table 2, 0.040 mgm. of histamine was recovered by a 36-hour extraction following a 24-hour extraction (this figure may be too high, as the color match with the standard was only fair, and some part of this color value was undoubtedly due to some substance not histamine).

When a known amount of histamine is added to a bacterial culture, the added histamine is removed quantitatively by a 24-hour extraction.

On the basis of these and other similar experiments, we have concluded that this extraction method will remove all but traces of histamine from bacterial cultures in 24 hours.

II. THE REMOVAL OF VOLATILE BASES AND AMYL ALCOHOL

As stated before, the acid extract from bacterial cultures will contain considerable amounts of ammonia and volatile amines, which react with the diazo reagent. They can be readily removed by boiling at a slightly alkaline reaction. This process at the same time removes amyl alcohol, which also interferes. Our procedure was as follows:

The acid extract was removed with a capillary pipette and the bulb rinsed with a few cubic centimeters of distilled water. To this, we added a determined amount of 2 per cent Na_2CO_3 solution, that would, after boiling, exactly neutralize to phenolphthalein the 10 cc. of 0.5 per cent sulfuric acid used in the extraction. Finally we added 0.5 cc. of 2 per cent borax solution. (This amount of borax does not interfere with the diazo reaction, though a larger amount will do so.) The fluid was then transferred to a special boiling tube (fig. 2) in which it was boiled vigorously until volatile bases no longer appeared in the vapors. To determine this point, we used as indicator a solution of Brom-Cresol-Green, which was adjusted with minimal acid until its color was a yellowish green. A platinum loopful of this indicator, held in the escaping vapors, will turn blue as long as

volatile bases are coming off. Five to 8 minutes of vigorous boiling usually suffices. The pH of the fluid should be near 9.2. The volume was then made up to 10 cc.; the fluid was now ready for the colorimetric determination.

The boiling tube shown in figure 2 was devised to prevent bumping, which is otherwise very troublesome. It is based upon the principle of creating a "hot spot" and preventing general superheating of the entire bottom. This principle has been

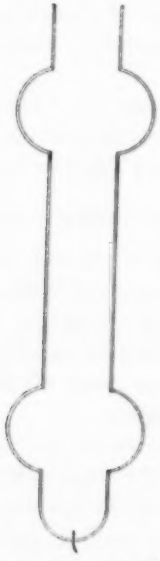

FIG. 2. BOILING TUBE WITH PLATINUM WIRE THROUGH BOTTOM

described by Moroney (1934) in connection with a quite different form of apparatus. The boiling tube was made from a thick walled Pyrex test tube, 200 by 25 mm. in size; a pin hole was blown through the bottom and a short piece of platinum wire passed through it. The hot glass was pinched down on the wire with heavy forceps, and the bottom worked in the flame until a smooth closure was obtained. The wire within the tube must be bare of glass. We have had no trouble with breakage

or leakage of these tubes. A small flame, passing through an 8 mm. hole in an asbestos gauze, is directed on the wire. Fluid can be boiled violently in this tube without any bumping. As some of our extracts frothed a good deal on boiling, the two expansions shown in the figure were blown in the tube.

Histamine solutions can be boiled for a long time in slightly alkaline reactions (pH 9.0 to 10.0) without loss of the amine. With greater alkalinity (pH 10.5 to 11.0 or more) some histamine is destroyed. We found that the removal of volatile bases by the evaporation of a neutral or alkaline solution was unsatisfactory, as there was always considerable loss of histamine. Only acid solutions of histamine can be evaporated to dryness without some loss.

III. THE COLORIMETRIC DETERMINATION OF HISTAMINE

We have followed exactly the method of Koessler and Hanke (1919a) for the diazo reaction and the colorimetric determination of histamine, which we have found very satisfactory. Our chief difficulty, at first, was that small quantities of histamine gave color values that were too low; after re-crystallizing the sulfanilic acid twice from hot water, this difficulty disappeared. For complete details, we refer the reader to Koessler and Hanke's article (1919a). Briefly, their technic is as follows:

Reagents for the test:

0.9 per cent sulfanilic acid in 10 per cent HCl.

5.0 per cent $NaNO_2$ solution.

1.1 per cent Na_2CO_3 solution.

The diazo reagent is prepared by measuring 1.5 cc. of the sulfanilic acid solution into a flask in an ice bath; then 1.5 cc. of the 5 per cent $NaNO_2$ solution are added. After 5 minutes, another 6.0 cc. of the $NaNO_2$ solution are added. In 5 more minutes, the reagent is made up to 50 cc. with cold distilled water. It is ready for use in 15 minutes.

The diazo reaction:

Five cubic centimeters of the 1.1 per cent Na_2CO_3 solution and $(1 - x)$ cubic centimeters of water are measured into a cylinder of the colorimeter (x is the volume of extract to be tested). Two cubic centimeters of the diazo reagent are added, within the space of 5 seconds, to the

alkali in the cylinder and mixed, noting the time to the second. (A stopwatch should be used.) In exactly 1 minute, x cc. of the test fluid are added and mixed. With histamine, the color is first yellow, then red; it is at its maximum in 4 to 5 minutes, after which it fades. The reading is made at the end of 5 minutes after the addition of the test fluid.

The volume x of test fluid should be chosen so that, if possible, the colorimetric reading falls between 5 and 20 mm. This can be determined by a rapid preliminary test: in quick succession, add 1 drop of the sulfanilic acid solution, 5 drops of the $NaNO_2$ solution, and 5 cc. of the Na_2CO_3 solution; then the test fluid is added drop by drop until the desired depth of color is obtained.

For standard solution we used the Congo-Red Methyl-Orange standard of Koessler and Hanke (1919a), which we also found very satisfactory. Two solutions are prepared: a Congo Red solution (2.500 grams of Grubler's Congo Red dissolved in 50 cc. of absolute alcohol, then water added to 500 cc.), and a Methyl Orange solution (0.500 grams of Methyl Orange in 500 cc. of water). The standard is made by adding 1.0 cc. of the Congo Red solution and 1.1 cc. of the Methyl Orange solution to about 250 cc. of water and diluting to 500 cc. This color standard deteriorates slowly and should be checked daily. For this purpose, a 1:10,000 solution of histamine di-hydrochloride, layered with toluene, is kept on hand; 0.25 cc. of this should give a reading of 18.7 mm.

To make the reading, the cup with the test solution is set at 20 mm.; the cup with the standard is moved until a match is found. A correction of 0.3 mm. is subtracted from the reading of the standard to allow for the color of the reagent. Tables given by Koessler and Hanke (1919a) enable one to calculate the concentration of histamine (as the di-hydrochloride) in the extract. Or the concentration may be calculated by the following formula:

$$\frac{\text{Reading of standard cup in mm.}}{\text{Volume of fluid tested in cc.}} \times 1\tfrac{1}{3} = \text{gammas of histamine di-hydrochloride per cc. of test solution.}$$

IV. THE PREPARATION OF HISTAMINE DI-PICRATE FROM THE ACID EXTRACT

As the identification of histamine is not complete unless a crystalline salt is prepared and identified by its melting point and mixed melting point with crystals of pure histamine salt, we prepared the histamine di-picrate from the acid extracts of cultures of all organisms that produced histamine in good yield. As the acid extracts are free from interfering substances, the di-picrates could be prepared and purified with ease.

It was our custom to grow each organism on several different kinds of media, and at several different temperatures. Consequently, when an organism was found positive for histamine, there were always ten or more tubes of extract available for preparing the di-picrate. After the colorimetric determinations were made, the remainders of such positive extracts were combined, adjusted to pH 6.0, and evaporated until the concentration of histamine was approximately 1 part in 1000. To every 9 cc. of this solution, 1 cc. of 10 per cent picric acid in methyl alcohol was added, and the mixture cooled over night. The di-picrates were re-crystallized from hot water 2 to 5 times until their melting points and mixed melting points were 239 to 241°C.

The dry histamine di-picrate can also be crystallized from absolute methyl alcohol. As the quantities were usually small, all crystallizations were done in small test tubes; the crystals were packed on the centrifuge and the supernatant fluids poured off. When the correct melting points were obtained, the di-picrates were dissolved in water and tested with the diazo reagent; in every case the characteristic red color was obtained.

DISCUSSION

When bacteria act upon histidine, it is quite possible that imidazols other than histamine may be produced. Inasmuch as these, if present in the acid extract, would produce a color like that of histamine, we felt it necessary to prepare and study such other derivatives of histidine, as follows:

Imidazol propionic acid. Found by Bertrand and Berthelot (1913) in cultures of their *Bacillus aminophilus.* It was prepared from imidazol lactic acid by the method of Knoop and Windaus (1905).

Imidazol lactic acid. Found in bacterial cultures by Hirai (1933). It was prepared by the method of Fränkel (1903).

Urocanic acid. Found in bacterial cultures by Raistrick (1917). It was prepared by his method from histidine by the action of *Salmonella paratyphi.*

Imidazol. Imidazol and methyl imidazol have not been described as occurring in bacterial cultures; but as they are the analogs of indol and scatol, they are possible bacterial metabolites. Imidazol was prepared from glyoxal sulfate by the method of Ruggli and Henzi (1929); the oxalate was prepared and crystallized from dilute acetone and dilute alcohol; m.p. 234°C.

4-Methyl imidazol. This was prepared by the method of Bernhauer (1929).

(4-Ethyl imidazol and 4-vinyl imidazol are also possible metabolites of histidine. We did not prepare these two substances. They should resemble 4-methyl imidazol in their properties.)

Imidazol ethyl alcohol. According to Ehrlich (1911) this substance is formed by the action of yeast upon histidine. It was prepared by the method of Windaus and Opitz (1911).

Solutions of these 6 imidazols, as well as of histidine and histamine, were studied and compared in the following ways:

1. When made alkaline and extracted in our apparatus in the usual way, only histamine, imidazol, methyl imidazol, and imidazol ethyl alcohol appear in the acid extract. Not the slightest trace of histidine, imidazol propionic acid, imidazol lactic acid, or urocanic acid will appear in the acid extract.

2. When coupled with sulfanilic acid, histamine gives an orange red color that exactly matches the Congo-Red Methyl-Orange standard of Koessler and Hanke (1919a). The color with histidine, urocanic acid, and imidazol ethyl alcohol is only slightly more orange. Imidazol lactic acid, imidazol propionic acid, and methyl imidazol yield a redder color that matches the Congo Red alone. Imidazol, on the other hand, gives a deep orange color, suggesting that, with the 4-position of the nucleus not occupied by a side chain, the coupling may take place at a different point than with the other imidazols.

3. When 1:1000 solutions of these 8 imidazols were treated with phosphotungstic and sulfuric acids, immediate precipitates

appeared with all but the imidazol lactic acid; with the latter, a small precipitate appeared on cooling. When these precipitates were removed by centrifugation, and the supernatant fluids neutralized and tested with the diazo reagent, strong colors developed in every case except that of histamine. *Of these bases, only histamine is completely precipitated by phosphotungstic acid.*

When 1:5000 solutions of these imidazols were tested with phosphotungstic and sulfuric acids, an immediate precipitate appeared only with histamine and imidazol ethyl alcohol. On standing in the refrigerator, small precipitates formed with histidine, urocanic acid, imidazol, and methyl imidazol. In dilutions of 1:10,000, a precipitate formed with histamine only.

Histamine, as Koessler and Hanke (1920) have shown, is completely precipitated by phosphotungstic acid. None of the other 7 imidazols here studied are completely removed. The presence of any of them in an extract can, therefore, be readily demonstrated by testing the supernatant fluid after phosphotungstic acid precipitation (it is necessary only to neutralize the acid). Conversely, if the supernatant fluid does not give the characteristic color with the diazo reagent, these other imidazols are absent. *We made it a practice to test every fluid that gave a histamine-like color in this way.*

As will be explained in the followed paper, in no case did the phosphotungstic acid supernatants of the acid extract contain any imidazols when the culture medium contained an organic compound of nitrogen (such as asparagine or peptone) in addition to histidine itself. But in cultures on a medium in which ammonium salts and nitrates were the only other source of nitrogen, such imidazols were frequently found (though in small quantities) in the supernatant fluids following phosphotungstic acid precipitation.

4. With Nessler's reagent, no precipitate is formed with histidine, imidazol propionic acid, imidazol lactic acid, or urocanic acid in a concentration of 1:1000. White precipitates are formed with histamine, imidazol, methyl imidazol, and imidazol ethyl alcohol. Nessler's reagent will produce a precipitate in a

1:700,000 solution of histamine di-hydrochloride. A modified Nessler's solution, made up with 16 per cent of Na_2CO_3 instead of the usual 10 per cent NaOH, will still precipitate histamine, though it will not precipitate ammonia.

5. Imidazol, methyl imidazol, and imidazol ethyl alcohol are soluble in ether; histamine and the imidazol acids are not.

6. Only histamine and urocanic acid yield picrates that are highly insoluble in water.

In addition to these imidazols, we tested the behavior of certain other substances that are likely to be present in the acid extracts, as follows:

1. Koessler and Hanke (1919c) encountered a base derived from histidine to which they ascribed the formula $HCNH_2$: $CNH_2CH_2CH_2NH_2$, though they did not isolate it. We encountered what is probably the same base; it is found most abundantly in cultures of the Friedlander-aerogenes group of organisms; it is also formed by other bacteria when histidine is the only source of both nitrogen and carbon. This base appears in the acid extracts. With Nessler's reagent, it gives a heavy white precipitate which soon turns yellow. It is incompletely precipitated with phosphotungstic and sulfuric acids. The color formed with the diazo reagent is pale yellow. The presence of this base can usually be neglected, as the color formed by it is so pale.

2. Tyramine might be formed in certain media and appear in the acid extract. As dilute solutions of tyramine are not precipitated by phosphotungstic acid, a test on the supernatant fluid would reveal it. We have never found, with the media used by us, any indications of tyramine.

3. If phenols or cresols are present in the culture, a very small fraction of them will appear in the acid extract. They would not be precipitated by phosphotungstic acid. We have never found phenols or cresols in recognizable quantities in the acid extracts of our cultures (which contained glucose and hence were acid in reaction). Hanke and Koessler (1924) also found that phenols and cresols were not formed in media that became acid.

4. Certain media, especially those that contain peptone and meat extract, will yield an acid extract containing a small amount of color-producing substance. Usually this color is pale yellow or

pale orange. When it is small in amount, it will not interfere with the determination of histamine. It is only necessary then to make a correction of the final reading for the medium. Media that require a large correction (more than 5 to 6 mm. per cubic centimeter) should be avoided.

The method described above has been used in over two thousand determinations of histamine, the results of which are reported in a following paper. This method requires a minimum of attention, labor, and reagents. An extract is obtained which contains histamine in a high state of purity; consequently, the color matches with the standard are very satisfactory. Only where the amount of histamine is very low (less than 5 gamma per cubic centimeter of culture) are the color matches likely to be "fair" rather than "excellent." Histidine and other imidazol acids never appear in the acid extract. With one medium only were histamine-like colors obtained that were in part due to some imidazol base other than histamine; that one medium contained, in addition to histidine, only ammonium salts and nitrates as a source of nitrogen. In all other media where other organic compounds of nitrogen were supplied, the characteristic color was due to histamine alone.

SUMMARY

1. A method for the quantitative extraction of histamine from small quantities of bacterial cultures is described.

2. A quantitative determination of histamine is made on this extract by the colorimetric method of Koessler and Hanke. Histamine di-picrate can also be readily prepared from this extract for positive identification.

3. Various substances that might interfere with the determination of histamine have been studied, and the means of recognizing and eliminating them indicated.

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THE PRODUCTION OF HISTAMINE IN BACTERIAL CULTURES

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In a previous paper (Eggerth, Littwin, and Deutsch, 1939) a simple and convenient method for the quantitative determination of histamine in bacterial cultures has been described. By this method, the cultures are made alkaline with sodium carbonate, then extracted in a special apparatus with a mixture of chloroform and amyl alcohol, the histamine passing into a layer of dilute sulfuric acid. Ammonia, volatile amines, and amyl alcohol are removed from this extract by boiling at pH 9.2. The histamine is determined colorimetrically by the method of Koessler and Hanke (1919a). The presence of histamine is then confirmed qualitatively by forming the di-picrate.

In the present investigation, a series of organisms has been studied to determine which of them are capable of producing histamine, and what factors influence histamine production.

A number of culture media were employed, as the composition of the medium profoundly affects the amount of histamine produced. A detailed report will be made of the following seven only. Histidine is an essential ingredient for all of them, for, with the exception of *Clostridium welchii*, none of the organisms studied were able to form histamine unless free histidine was present.[1]

The first five media were adjusted to pH 7.6 and autoclaved; then enough sterile concentrated glucose solution was added to

[1] Suzuki and Joshimura (1909) have shown that infusions of the flesh of certain sea fish are rich in free histidine. In the present investigation, it was found that many organisms produced high yields of histamine on a medium consisting of fresh mackerel infusion with asparagine or peptone and glucose, with no added histidine.

give a concentration of 1 per cent. Medium 6 was also adjusted to pH 7.6, but 2 per cent of glycerol was added instead of the glucose. The media were then distributed in sterile tubes, 12 cc. per tube.

No. 1. Ammonium-nitrate-histidine-glucose medium:

300 cc. H_2O
0.3 gram histidine di-hydrochloride
0.3 gram $(NH_4)_2SO_4$
0.6 gram $NaNO_3$
0.6 gram $Na_2HPO_4 \cdot 12H_2O$
0.3 gram KCl
0.05 gram $CaCl_2$
0.05 gram $MgSO_4$

Many organisms failed to grow on this medium. A control determination made on the uninoculated medium gave no color with the diazo reagent.

No. 2. Asparagine-histidine-glucose medium:

300 cc. H_2O
1.0 gram asparagine
0.3 gram histidine di-hydrochloride
0.6 gram $Na_2HPO_4 \cdot 12H_2O$
0.3 gram KCl
0.05 gram $CaCl_2$
0.05 gram $MgSO_4$

A control determination on the uninoculated medium gave no color with the diazo reagent.

No. 3. Asparagine-histidine-cysteine-glucose medium. This has the same formula as medium 2, but with the addition of 0.3 gram cysteine hydrochloride.

No. 4. Egg-yolk-asparagine-histidine-glucose medium. Seven parts of distilled water were added to 1 part of fresh egg yolks and stirred. The mixture was heated in the Arnold sterilizer for 1 hour and filtered.

300 cc. egg yolk infusion
0.3 gram histidine di-hydrochloride
1.0 gram asparagine
0.6 gram $Na_2HPO_4 \cdot 12H_2O$
0.3 gram KCl
0.05 gram $MgSO_4$

A control determination on the uninoculated medium gave a color reading of 0.3 mm. per cubic centimeter. This medium gave excellent growth with most organisms, and the yields of histamine were high.

No. 5. Meat-extract-peptone-histidine-glucose medium:

300 cc. H_2O
1.2 grams meat extract
3.0 grams peptone (Parke Davis)
0.3 gram histidine di-hydrochloride

A blank determination on the uninoculated medium gave a color reading of 5.6 mm. per cubic centimeter which matched the histamine standard fairly well.

No. 6. Meat-extract-peptone-histidine-glycerol medium. This medium has the same formula as the preceding one, except that the carbohydrate added is 2 per cent glycerol.

No. 7. Histidine alone, with no other source of nitrogen and no carbohydrate:

300 cc. H_2O
0.3 gram histidine di-hydrochloride
1.2 grams $Na_2HPO_4 \cdot 12H_2O$
0.3 gram KCl
0.05 gram $CaCl_2$
0.05 gram $MgSO_4$

Acetic acid or sodium hydroxide were added to obtain the desired pH. Little or no growth is to be expected in this medium; it was used to determine histamine production by "resting" bacteria. A blank test on the uninoculated medium gave no color with the diazo reagent.

In calculating the amount of histamine produced in a culture, a correction was made in each case for the blank for that medium, in addition to a correction of 0.3 mm. for the color of the reagent.

I. THE RATE OF HISTAMINE PRODUCTION IN BACTERIAL CULTURES

Koessler and Hanke (1919b) determined the rate of histamine production by *Escherichia coli* in a medium containing only

inorganic salts and glycerol in addition to histidine. In their experiment, histamine formation took place very slowly; none was formed in 2 days; in 5 days, only 3.3 per cent of the available histidine had been converted to histamine; in 10 days, 24.4 per cent; and in 40 days, 83.5 per cent.

When a richer medium is used, histamine production is much more rapid, as is shown in table 1.

Similar experiments with other organisms and other media show that on the richer media considerable histamine is formed

TABLE 1

Rate of the production of histamine by Shigella dysenteriae St. on medium 5 (meat extract-peptone-histidine-glucose) at 31°C.

	HISTAMINE PRODUCED (AS THE DIHYDROCHLORIDE)	HISTIDINE CONVERTED TO HISTAMINE
	mgm. per cc.	*per cent*
1-day culture	0.094	11.6
2-day culture	0.191	23.6
5-day culture	0.417	51.6
11-day culture	0.444	55.0
15-day culture	0.463	57.3
23-day culture	0.460	56.9
30-day culture	0.458	56.7

during the first 24 hours; that most of the production takes place within 5 days, and that the maximum yield is obtained in about 2 weeks.

II. THE EFFECT OF THE pH ON THE PRODUCTION OF HISTAMINE

The effect of the pH was tested in a variety of ways. The most satisfactory procedure was as follows: A small amount of glucose (0.3 per cent) was added to the medium. Eight to 10 hours after inoculation, when active growth had started, the cultures were adjusted to the desired pH by the addition of sterile 10 per cent acetic acid or 4 per cent sodium hydroxide. During the first 48 hours, the pH of a culture changes rapidly, and the pH must be adjusted every 6 to 8 hours; after that, two or even one daily adjustment suffices. To determine the pH,

large loopfuls of the cultures were mixed with drops of indicator on a spot plate; the colors were then compared with those produced in a similar way by known buffer solutions.

The results thus obtained with several histamine-producing organisms are shown in table 2. This table shows that, with two exceptions, the maximum yields of histamine are obtained at pH 5.0 to 5.5. In no case (except with *Aerobacter aerogenes*) was there appreciable histamine formation at a pH more alkaline than 6.2.

TABLE 2

The effect of the pH on the histamine production on medium 5 (meat extract-peptone-histidine, but with 0.3 per cent glucose)

ORGANISM	TEMPERATURE OF INCUBATION	pH 4.5	pH 5.0	pH 5.3	pH 5.5	pH 5.7	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
	°C.										
Escherichia coli Sta.	26	0.008	0.016	0.016	0.021	0.037	0.070	0.013	0	0	0
Escherichia coli Ev.	31	0.107	0.168	0.053	0.044	0.032	0.010	0.002	0	0	0
Escherichia coli Har.	37	0.065	0.123	0.098	0.098	0.068	0.020	0.005	0	0	0
Salmonella schottmuelleri Old.	31	0.157	0.389	0.364	0.304	0.276	0.065	0.034	0	0	0
Shigella dysenteriae St.	31	0.357	0.432	0.448	0.245	0.242	0.245	0.020	0	0	0
Shigella paradysenteriae Rya.	31	0.061	0.133	0.157	0.120	0.088	0.073	0.020	0	0	0
Shigella alkalescens Kau.	31	0.123	0.266	0.197	0.187	0.136	0.081	0.015	0	0	0
Eberthella typhi Mt. S.	37	0.107	0.264	0.357	0.352	0.243	0.165				
Aerobacter aerogenes Hul.	26	0.136	0.211	0.485	0.501	0.488	0.485	0.485	0.488	0.272	0.048

The results are recorded as mgm. of histamine di-hydrochloride per cubic centimeter of culture. The time of incubation was 14 days. The temperatures selected were those that are optimal for histamine production by that organism.

The two organisms in table 2 that gave unusual results are *Escherichia coli* Sta. and *Aerobacter aerogenes* Hul. *E. coli* Sta., which is a very efficient producer of histamine on other media (table 3) has consistently given low values for the meat-extract-peptone-histidine-glucose medium, which apparently contains some inhibitory factor which may also be responsible for the somewhat atypical pH effect. *Aerobacter aerogenes* Hul., and other histamine producers of this species, differ from all other histamine-forming organisms in being uninfluenced by the pH over a wide range. Only at the extremes (pH 5.0 to 5.5, and 7.5 to 8.0) is there any decrease in histamine production.

Koessler and Hanke (1919b) concluded, from their study of

Escherichia coli, that an acid reaction was indispensable for histamine production. Bertrand and Berthelot (1913), Mellanby and Twort (1912–13) and Jones (1918), on the other hand, have described organisms which produce histamine in alkaline reactions only, never in the presence of glucose. A search was made for such organisms, using the technic employed by these authors, but without success. As Jones succeeded only once in 50 attempts, such organisms cannot be common.

III. THE EFFECT OF THE TEMPERATURE AND THE COMPOSITION OF THE MEDIUM ON HISTAMINE PRODUCTION

Table 3 shows the effect of these two factors on histamine production by several representative organisms.

Temperatures above 37°C. depress histamine formation by all of the bacteria tested. In most cases, temperatures of 26°C. or less are also unfavorable. With some organisms (such as *Salmonella enteriditis* V and some not given in table 3) variations between 37°C and 26°C. have very little effect. In most instances, however, the temperature effect is very striking. An extreme case is that of *Escherichia coli* Sta., which produces over 40 times as much histamine at 26°C. as at 37°C. on the ammonium-nitrate-histidine-glucose medium. Differences of 3- or 4-fold in the histamine yield with a variation of only a few degrees of temperature are common.

Different species and even different strains of the same species respond differently to temperature variations. Thus, most strains of *Escherichia coli* have their optimum for histamine production at 31° to 26°C., but others at 37°C. (such as *Escherichia coli* Har., table 3). All of the *Salmonella* strains studied have their optimum at 34° to 31°C.; 7 strains of *Eberthella typhi* at 37° to 34°C.; 4 strains of *Shigella* at 31° to 26°C.; 5 strains of *Aerobacter aerogenes* at 26°C.; *Clostridium welchii* and *Bacteroides varius* at 37° to 34°C.; and *Bacteroides ovatus* at 31°C.

The composition of the culture fluid has a marked effect upon histamine production, as has been recognized by Bertrand and Berthelot (1913) and by Hanke and Koessler (1922). In these

TABLE 3

Effect of the temperature and the composition of the medium on histamine production

ORGANISM	TEMPERATURE	MEDIUM 1. AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2. ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3. ASPARAGINE-HISTIDINE-CYSTEINE-GLUCOSE	MEDIUM 4. YOLK INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5. MEAT EXTRACT-PEPTONE-HISTIDINE-GLUCOSE	MEDIUM 6. MEAT EXTRACT-PEPTONE-HISTIDINE-GLYCEROL
	°C.						
	41				0.009	0.011	
Escherichia coli Sta.	37	0.010	0.032	0.157	0.055	0.046	0.537
	34	0.185	0.331	0.840	0.480		
	26	0.425	0.451	0.629	0.637	0.126	0.633
	20	0.041	0.440	0.580	0.522	0.078	0.118
	41			0.013	0.025	0.021	
Escherichia coli Har.	37	0.003	0.017	0.058	0.175	0.166	0.033
	31			0.032	0.058	0.141	
	26	0.002	0.001	0.023	0.029	0.110	
	37	0.014	0.104	0.007	0.267	0.124	
Escherichia coli Tem.	31	0.061	0.216	0.065	0.492	0.151	0.041
	26	0.064	0.272	0.065	0.645	0.197	
	37	0.002	0.001	0.014	0.138	0.336	0
Salmonella schottmuelleri Old.	34	0.003	0.005	0.012	0.268	0.288	0
	31	0.003	0.009	0.021	0.264		
	26	0.004	0.011	0.016	0.305	0.240	0
	37	0	0	0.007	0.200	0.135	0
Salmonella enteriditis V.	34	0	0	0.008	0.211	0.129	0.010
	31	0	0	0.007	0.352	0.236	0.015
	26	0	0	0.009	0.261	0.196	
	41				0.027	0.111	
	37				0.298	0.303	0.010
Eberthella typhi Mt. S.	34	No growth			0.341	0.316	0.008
	31				0.200	0.283	0.005
	26				0.099	0.181	
	37	0	0.007	0.016	0.267	0.234	0.022
Shigella dysenteriae St.	34			0.026	0.481	0.362	
	31	0	0.034	0.037	0.498	0.403	0.003
	26			0.035	0.391	0.411	

TABLE 3—*Concluded*

ORGANISM	TEMPERATURE	MEDIUM 1. AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2. ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3. ASPARAGINE-HISTIDINE-CYSTEINE-GLUCOSE	MEDIUM 4. TOLE INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5. MEAT EXTRACT-PEPTONE-HISTIDINE-GLUCOSE	MEDIUM 6. MEAT EXTRACT-PEPTONE-HISTIDINE-GLYCEROL
	°C.						
Shigella paradysenteriae Rya.	37	0.013	0.069	0.076	0.560	0.133	0.388
	31	0.080	0.347	0.399	0.587	0.111	0.356
	26	0.102	0.475	0.644	0.651	0.230	
Shigella alkalescens Kau.	37	0.024	0.049	0.104	0.352	0.060	0.590
	31	0.072		0.544	0.803	0.136	0.451
	26			0.651	0.597	0.206	0.060
Aerobacter aerogenes Hul.	41			0	0	0	0
	37	0.001	0.215	0.092	0.011	0.015	0.304
	31	0.025	0.381	0.500	0.512	0.405	0.325
	26	0.042	0.466	0.582	0.619	0.243	0.284
	20	0.003	0.485	0.419	0.275		
Clostridium welchii Ti.	37	No growth			0.165*	0.391	
	31				0.157*	0.251	
Bacteroides ovatus	37				0.076*	0.091	
	31				0.296*	0.267	

Results are expressed in milligram of histamine di-hydrochloride per cubic centimeter of culture. The time of incubation was 14 days.

* Cysteine hydrochloride was added to this medium in a concentration of 1:1000.

experiments, the lowest yields usually occurred with the ammonium-nitrate-histidine-glucose medium. This is partly explained by the fact that growth here is often sparse or lacking; the pH attained is usually 5.6 to 6.0, which is not optimal. With this medium, a small quantity of imidazol bases other than histamine may appear in the acid extract (see table 6).

When an organic compound of nitrogen, such as asparagine, is supplied in addition to the histidine, the histamine yield is usually increased. Such an increase was observed by Hanke and

Koessler (1922) with several amino acids. This increase is in part due to the more favorable pH obtained (pH 5.0 to 5.5 when glucose is present). In many cases, the addition of cysteine to the asparagine medium still further increases histamine production; with one organism, however (*Escherichia coli*, Tem.), the addition of cysteine greatly diminished the yield.

When egg-yolk infusion was added to the medium, histamine production was in most cases greatly stimulated. In many instances, especially with the anaerobes and with all strains of *Eberthella typhi*, the addition of cysteine still further augmented the yield of histamine.

The meat-extract-peptone-histidine-glucose medium was rather variable in its effect on histamine production. In many cases, it was as good or better than other media; while with other organisms it gave comparatively low yields.

When glycerol was used instead of glucose, as in the meat-extract-peptone-histidine-glycerol medium, the production of histamine was usually very low. This is probably due to the fact that these organisms produce little or no acid from glycerol, hence the pH remains too alkaline for histamine production. But in 5 instances (3 strains of *Escherichia coli*, *Shigella paradysenteriae* Rya., and *Shigella alkalescens* Kau.) the production of histamine was considerably increased when glycerol was substituted for glucose (tables 3 and 4). Also, with *Aerobacter aerogenes*, there was a marked difference in favor of the glycerol, but only at the higher temperatures. With the 5 strains mentioned above, the pH with the glycerol medium ran between 5.7 and 6.2, reactions which one would expect to be less favorable for histamine production than the pH 5.0 to 5.3 obtained on the glucose medium. The results indicate that for these strains at least, the carbohydrate has some other effect than that of stimulating growth and providing a favorable acidity.

The addition of 5 per cent of serum to these media made very little difference in the histamine yield. As a rule, the addition of serum slightly diminished histamine production; in a few cases, it slightly increased the yield.

TABLE 4

Production of histamine by bacteria on media of different composition

ORGANISM	TEMPERATURE	MEDIUM 1. AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2. ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3. ASPARAGINE-CYSTEINE-HISTIDINE-GLUCOSE	MEDIUM 4. YOLK INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5. MEAT EXTRACT-PEPTONE-HISTIDINE-GLUCOSE	MEDIUM 6. MEAT EXTRACT-PEPTONE-HISTIDINE-GLYCEROL
	°C.						
Escherichia coli Sta.*	26	0.425	0.452	0.629	0.637	0.126	0.633
Escherichia coli Har.*	37	0.003	0.017	0.058	0.175	0.166	0.033
Escherichia coli O'L	26	0.002	0.002	0.003	0.019	0.097	0.005
Escherichia coli Sch	26	0	0.003	0.012	0.044	0.088	0
Escherichia coli Tem.*	26	0.064	0.272	0.065	0.645	0.197	
Escherichia coli Mal	31	0	0	0	0.025	0.053	0
Escherichia coli Ort.*	37	0	0	0	0.002	0.154	0.268
Escherichia coli Kie	37	0	0.067	0.106	0.121		
Escherichia coli Bro.*	37	0	0.020	0.016	0.205	0.107	0.120
Escherichia coli Rak	37	0	0.003	0.005	0.027		
Escherichia coli Pen	26	0	0.002	0.002	0.072	0.273	0.039
Escherichia coli Ev	26	0	0.001	0.006	0.024	0.188	0.030
Escherichia coli Jon	26	0	0.007	0.006	0.051		
Escherichia coli H 1	34				0.307	0.128	
Escherichia coli H 2	34			0	0.041	0.110	
Escherichia coli Ton	34				0.213		
Escherichia coli McC	34				0.053		
Escherichia coli Mer	34				0.212		
Salmonella paratyphi St	31	0	0.002	0.027	0.109		
Salmonella paratyphi Frl.*	31	0	0	0.009	0.267	0.142	0.016
Salmonella schottmuelleri Old.*	34	0.003	0.005	0.012	0.268	0.288	0
Salmonella schottmuelleri 222*	31			0.007	0.352	0.324	0.006
Salmonella schottmuelleri Rak.*	31	0	0	0	0.190		
Salmonella enteriditis V.*	31	0	0	0.007	0.352	0.236	0.015
Salmonella enteriditis McK	31			0.009	0.101		
*Salmonella suis**	31			0.002	0.147	0.306	0.004
*Salmonella aertrycke**	31			0	0.152	0.203	0.017
Eberthella typhi Pfe.*	37				0.087	0.151	0
Eberthella typhi Sch.*	37				0.188	0.090	0.005
Eberthella typhi Mt. S.*	37				0.298	0.303	0.010
Eberthella typhi Mor	37	No growth			0.064	0.082	0
Eberthella typhi Spa	37				0.009	0.115	0
Eberthella typhi Bra	37				0.007	0.129	0
Eberthella typhi Dan.*	37				0.090	0.200	0.002

* Crystals of histamine di-picrate, m.p. 238–241°C. were prepared from the acid extracts.

TABLE 4—*Concluded*

ORGANISM	TEMPERATURE	MEDIUM 1. AMMONIUM-NITRATE-HISTIDINE-GLUCOSE	MEDIUM 2. ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 3. ASPARAGINE-CYSTEINE-HISTIDINE-GLUCOSE	MEDIUM 4. YOLK INFUSION-ASPARAGINE-HISTIDINE-GLUCOSE	MEDIUM 5. MEAT EXTRACT-PEPTONE-HISTIDINE-GLUCOSE	MEDIUM 6. MEAT EXTRACT-PEPTONE-HISTIDINE-GLYCEROL
	°C.						
Shigella dysenteriae St.*	31	0	0.034	0.037	0.498	0.403	0.003
Shigella paradysenteriae Rya.*	26	0.102	0.475	0.644	0.651	0.230	0.356
Shigella paradysenteriae Son.	26	0	0	0	0.097	0.047	
Shigella alkalescens Kau.*	31	0.072		0.544	0.803	0.136	0.451
Aerobacter aerogenes Hul.*	26	0.042	0.466	0.582	0.619	0.243	0.284
Aerobacter aerogenes Woo.*	26	0.093			0.338	0.668	0.700
Aerobacter aerogenes Sm.	26				0.041	0.020	0.078
Aerobacter aerogenes Mc.	26			0.027	0.141	0.184	0.067
Aerobacter aerogenes El.	31				0.272	0.144	0.034
Clostridium welchii Ti.*	37	No growth			0.165†	0.391	
Clostridium welchii Eg.	34				0.120†	0.251	
Clostridium welchii Br.	34				0.120†	0.167	
Clostridium welchii Ca.	34				0.162†	0.275	
*Bacteroides ovatus**	31				0.296†	0.275	
*Bacteroides varius**	37				0.022†	0.256	

† Cysteine hydrochloride was added to a concentration of 1:1000. Results are expressed in milligram of histamine di-hydrochloride per cubic centimeter of culture. The time of incubation was 14 days.

IV. THE ORGANISMS THAT WERE FOUND TO PRODUCE HISTAMINE

In table 4, all of the organisms that were found to produce histamine have been listed. Most of them were tested at several different temperatures; in this table, only the yields for one temperature are given. This was not always the optimum, as the optimum temperature was not determined in every case.

One is impressed by the fact that every organism in table 4 has its habitat in the intestinal tract; not one non-intestinal organism produced histamine. The converse, however, is not true, for many species of intestinal bacteria do not produce histamine.

Every strain of *Escherichia coli* that was tested produced

some histamine. This was likewise true of every strain of the genus *Salmonella* and of every strain of *Eberthella typhi*. On the other hand, of the 8 strains of *Shigella* that were investigated, only the 4 given in table 4 were positive; and of 14 strains of *Aerobacter aerogenes*, only 5 produced histamine. Four strains only of *Clostridium welchii* were studied; all were positive. Only one strain each of *Bacteroides ovatus* and *Bacteroides varius* were available.

V. HISTAMINE PRODUCTION BY "RESTING" BACTERIA

Medium 7, containing only histidine and salts, was employed in these experiments. As little or no growth can take place in this fluid, very heavy inoculations were made. Young agar cultures were washed twice with sterile distilled water, and enough organisms were added to the medium to give a density of about 2 billion bacteria per cubic centimeter.

TABLE 5

Histamine production by resting bacteria

ORGANISM	HISTAMINE DI-HYDROCHLORIDE PRODUCED	HISTIDINE CONVERTED
	mgm. per cc.	*per cent*
Escherichia coli Sta	0.694	85.9
Escherichia coli Har	0	0
Salmonella schottmuelleri 222	0	0
Eberthella typhi Mt. S	0	0
Shigella dysenteriae St	0	0
Shigella paradysenteriae Rya	0.437	54.1
Shigella alkalescens Kau	0.778	96.3
Aerobacter aerogenes Hul	0	0

The temperature was 34°C., and the time of incubation was 7 days.

In one experiment (table 5) the medium was brought to pH 5.2 with acetic acid; it is therefore well buffered at this pH.

All of the organisms shown in table 5 will produce abundant histamine on other media. Yet only 3 of these 8 organisms formed histamine under these conditions; the same 3 will form histamine in the ammonium-nitrate-histidine-glucose medium. The 5 negative organisms of table 5 partially converted the

histidine to another base that appeared in the acid extracts; this base does not contain the imidazol nucleus, for only a pale yellow color is produced when it is coupled with sulfanilic acid. It is apparently identical with a base encountered by Koessler and Hanke (1919b) to which they ascribed the formula $HCNH_2{:}CNH_2CH_2CH_2NH_2$. Imidazol bases other than histamine did not appear in the acid extracts.

VI. THE PRODUCTION OF IMIDAZOL BASES OTHER THAN HISTAMINE

As stated before, imidazol bases other than histamine appeared in the acid extracts only when the cultures were made on medium

TABLE 6

Production of histamine and of extractable imidazol bases other than histamine on the ammonium-nitrate-histidine-glucose medium

ORGANISM	HISTAMINE	IMIDAZOL BASE, NOT HISTAMINE
Escherichia coli Sta	0.299	0.025
Escherichia coli Kier	0	0.012
Escherichia coli Tem	0.061	0.012
Shigella paradysenteriae Rya	0.057	0.015
Shigella alkalescens Kau	0.072	0.016

The time of incubation was 14 days; the temperature, 31°C.

The results in both columns are expressed as histamine di-hydrochloride in milligrams per cubic centimeter.

1. In this medium, ammonium salts and nitrates are the only sources of nitrogen beside the histidine, and glucose is present. When the amount of these bases is small (as is invariably the case), they will not be precipitated from the acid extracts by phosphotungstic acid (Eggerth, Littwin, and Deutsch, 1939), whereas the histamine will be completely removed. If the precipitate is dissolved in sodium carbonate solution, and the supernatant fluid is neutralized, both fractions can be assayed with the diazo reagent in the usual way. The results of several such tests are given in table 6.

This table shows that the total amounts of non-histamine imidazol bases in the acid extract were always small. For

this reason, no attempt was made to isolate and identify them. When treated with the diazo reagent, their color was redder than that of the histamine standard, suggesting that they might be methyl imidazol or some homologue such as ethyl or vinyl imidazol.

VII. ORGANISMS THAT DO NOT PRODUCE HISTAMINE

The following bacteria were tested on two or more media each, and at two different temperatures, and were found negative for histamine.

	Number of strains
Proteus vulgaris	6
Alcaligenes fecalis	2
Shigella paradysenteriae	4
Aerobacter aerogenes	9
Klebsiella pneumoniae	3
Klebsiella ozaenae	1
Klebsiella rhinoscleromatis	1
Vibrio comma	1
Vibrio metchnikovi	1
Brucella abortus	1
Brucella melitensis	1
Pseudomonas aeruginosa	2
Neisseria intracellularis	3
Neisseria gonorrheae	2
Neisseria flava	1
Neisseria catarrhalis	1
Neisseria crassus	1
Streptococcus hemolyticus	3
Streptococcus viridans	1
Enterococcus	6
Diplococcus pneumoniae	3
Staphylococcus aureus	3
Corynebacterium diphtheriae	1
Bacillus subtilis	2
Bacillus mycoides	1
Bacillus mesentericus	1
Bacillus megatherium	1
Lactobacillus acidophilus	2
Clostridium tetani	2
Clostridium putrificum	1
Clostridium histolyticum	1
Clostridium bifermentans	1
Bacteroides bifidus	4
Bacteroides pseudoramosus	3

	Number of strains
Bacteroides aerofaciens	3
Bacteroides biformis	3
Bacteroides avidus	2
Bacteroides limosus	1
Bacteroides catenaformis	2
Bacteroides lentus	3
Bacteroides gulosus	3
Bacteroides thetaiotaomicron	3
Bacteroides variabils	3
Bacteroides uniformis	3
Bacteroides vulgatus	4
Bacteroides distasonis	3
Bacteroides exiguus	1
Bacteroides vescus	1
Bacteroides insolitus	1

Where organisms did not grow well on the usual media, special media were used. Thus, 5 per cent of serum was added to cultures of the *Neisseria*, *Streptococcus*, and *Diplococcus* genera; and *Lactobacillus acidophilus* was cultivated in a milk histidine medium.

DISCUSSION

The only other investigations in which a large number of strains and species of organisms were studied have been those of Hanke and Koessler (1922) and of Koessler, Hanke, and Sheppard (1928). Hanke and Koessler studied 62 strains (29 of these were *Escherichia coli*) on a medium containing only histidine, inorganic salts, and glycerol. Six of these strains—all of them *Escherichia coli*—formed histamine. No positive results were obtained with any member of the *Salmonella*, *Eberthella*, or *Shigella* genera. Koessler, Hanke, and Sheppard studied 223 strains from 94 species on a medium containing meat extract, peptone, histidine, whole blood, and glycerol. (These authors preferred glycerol to glucose because, when glucose was used, troublesome interfering substances were extracted by their method.) In this investigation, 9 histamine producing organisms were found; 2 were *Escherichia coli*, 1 was a *Salmonella enteriditis*, 1 was a *Salmonella schottmuelleri*, and 5 were *Salmonella morgani*. In the same series, 7 other strains of *Escherichia coli* and 42

other strains of *Salmonella* were negative, as were also all strains of *Eberthella*, *Shigella*, and *Aerobacter*.

In the present investigation, histamine was formed by every one of the 18 strains of *Escherichia coli* tested; likewise every one of 9 strains of *Salmonella* (from 5 species), and every one of 7 strains of *Eberthella typhi*. Four out of 8 strains of *Shigella* were positive, as well as 5 out of 14 strains of *Aerobacter aerogenes*. Organisms from all of these genera were active producers of histamine, often converting from 30 to 100 per cent of the histidine to the amine. There can be no doubt that the color-producing substance formed by these organisms actually was histamine, as the crystalline di-picrates were prepared in most cases from the acid extracts (table 4).

There are several reasons why many more histamine-positive organisms were found than in the above mentioned investigations. The most important one is that glucose was employed in the media instead of glycerol. When glucose is supplied, the favorable pH of 5.0 to 5.5 is usually obtained, whereas most organisms do not produce enough acid from glycerol to permit histamine formation. Tables 3 and 4 show the striking effect of substituting glucose for glycerol.

Very often the temperature of incubation is an important contributory factor. Hanke and Koessler (1922) and Koessler, Hanke, and Sheppard (1928) incubated their cultures at 37.5°C., which is not, in most cases, the optimal temperature for histamine production.

Hanke and Koessler (1922) employed a medium that contained no organic source of nitrogen except histidine itself. Tables 3 and 4 show that such a medium is usually unfavorable to histamine production, and that very few organisms will be positive when it is used.

SUMMARY

1. Using a simplified technic previously described (Eggerth, Littwin, and Deutsch, 1939) histamine production has been determined in cultures of 49 strains of bacteria, belonging to 14 species of 7 genera. These organisms all have their habitat in the intestinal tract.

2. With the exception of *Clostridium welchii*, all of these organisms require free histidine for histamine formation.

3. In a favorable medium, histamine production begins within 24 hours and continues rapidly for 4 to 5 days, after which the rate of production decreases.

4. For most organisms, the optimal pH for histamine production is pH 5.0 to 5.5, and no histamine is produced at reactions more alkaline than pH 6.5. However, the histamine forming strains of *Aerobacter aerogenes* will produce this amine at any pH between 5.0 and 8.0.

5. The temperature of incubation markedly affects histamine formation. Temperatures higher than 37°C. and lower than 26°C. are usually unfavorable. Between these limits, the optimal temperature varies a great deal with different organisms.

6. The yield of histamine is determined also by the composition of the culture medium. Where only inorganic compounds of nitrogen are supplied, in addition to histidine, the yields of histamine are usually low, and some of the histidine may be converted to other imidazol bases. The addition of amino acids, such as asparagine and cysteine, or of peptone, or of egg yolk or meat infusion, increases histamine production. The nature of the added carbohydrate is also important, chiefly because of the effect of the pH of the culture.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL NEW YORK STATE BRANCH

VETERINARY COLLEGE, CORNELL UNIVERSITY, NOVEMBER 19, 1938

THE SEVENTH EDITION OF STANDARD METHODS FOR THE EXAMINATION OF DAIRY PRODUCTS. *Robert S. Breed*, N. Y. State Agricultural Experiment Station, Geneva.

This well-known report is soon to be issued in a new edition as approved by the American Public Health Association at their Kansas City meeting, October 24–28, 1938. The changes affect the composition of the agar used in making agar plate counts, and the temperature of incubation used for these plates. It is required that properly constructed incubation chambers be used and that the temperature be held at a point where it does not exceed 37°C nor fall lower than 35°C.

The use of microscopic examination of pasteurized milk as a means of checking the presence of large numbers of bacteria, either living or dead, is also emphasized in this new report.

THE UDDER STREPTOCOCCI OF EIGHTEEN DAIRY HERDS. *Jean Ferguson*, Veterinary College, Cornell University, Ithaca.

A mastitis-survey of 18 dairy herds has included the examination of quarter milk samples for the presence of streptococci. For this purpose 0.1 cc. of milk was plated on 8 per cent horse-blood agar. One hundred thirty-eight (21 per cent) of the 655 cows were infected with streptococci. Of the 229 cultures isolated from as many quarters, 160 (70 per cent) were *Streptococcus agalactiae;* 16 (7.0 per cent) were *Streptococcus dysgalactiae;* 25 (10.8 per cent) were *Streptococcus uberis;* 13 (5.6 per cent) were of other types, and on the remaining 15 cultures (6.5 per cent) no tests were made.

All of the *Streptococcus uberis* and *Streptococcus dysgalactiae*, 52 of the *Streptococcus agalactiae* cultures, and 11 of the 13 streptococci of other types produced green discoloration of blood agar. The remainder of the *Streptococcus agalactiae* cultures were either indifferent to blood or produced narrow- or broad-zone hemolysis. This broad-zone hemolysis was distinguished from the hemolysis of two Group C and E cultures by an outer zone of hemolysis.

In those herds in which *Streptococcus agalactiae* was present, it was the predominating organism and the infection tended to be widespread. Infections due to other streptococci appeared to be sporadic in origin.

Only in the most heavily infected herd could streptococci have been observed in a Breed smear of the mixed milk.

FOUR TEMPERATURE OPTIMA OF BACTERIA. *Leslie Dorn*, N. Y. State College of Agriculture, Ithaca.

THE INHIBITION OF BACTERIOPHAGE BY PHOSPHOLIPIDS. *Clara H. Williams*,

Leslie A. Sandholzer and George Packer Berry, University of Rochester, School of Medicine and Dentistry, Rochester.

Phospholipids, from bacterial and non-bacterial sources, and cholesterol were tested for an inhibitory action on a bacteriophage (C13, Burnet) which lyses a variety of coliform organisms. The plaque-count method in semi-solid agar was employed.

Using two bacteriophage-susceptible strains of *Escherichia communior* as test organisms, it was found that lecithin from soybeans and sphingomyelin from sheep brains failed to inhibit the bacteriophage in concentrations up to 1 per cent. However, two samples of cephalin, one from beef livers and the other from sheep brains, were markedly inhibitory in concentrations of 0.2 per cent. This action persisted after the lecithin had been mixed with the samples of cephalin in a proportion of 5:1.

Phospholipids from the blood plasma of a dog exerted no inhibition. A sample of purified cholesterol, however, was strongly inhibitory in a concentration of 1 per cent.

The two strains of *Escherichia communior*, mentioned above, and a strain of *Escherichia coli* which was resistant to the bacteriophage, were used as source materials for the preparation of bacterial phospholipids. The phospholipids from all three strains were markedly inhibitory, a finding which indicates that the inhibitory activity is not specific in regard to susceptibility or resistance to bacteriophagy.

FURTHER EXPERIMENTS IN THE DISINFECTION OF SEEDS. *A. W. Hofer and H. C. Hamilton*, New York State Agricultural Experiment Station, Geneva.

Further studies have been made to discover a disinfectant selective for the contaminating organisms of seeds.

Among the agents used, two were unusual. One was the commonly used chlorine, but in the form of hypochlorous acid; the other was a war gas, iodoacetone, selected for its penetrating properties. (Chlorine is ordinarily used as a hypochlorite; when this is treated with a weak acid, hypochlorous acid is liberated—an agent many times more potent than the relatively stable commercial preparations. Iodoacetone is prepared from chloracetone and is water soluble.)

Many experiments were made in this research to improve methods. Seeds were soaked in water before applying the disinfectant. In other experiments the coats were removed from peas and soybeans to obtain a clue to the location of contaminants. It required in both cases stronger solutions to obtain results equivalent to those for seeds not previously soaked. Seeds, apparently disinfected, were crushed and returned to the beef broth where bacterial growth subsequently occurred in almost every tube. Evidently the bacteria were in the interior of the seed.

CONTAMINATION OF DRINKING GLASSES BY STREPTOCOCCI. *Leo A. Dick*, New York State Agricultural Experiment Station, Geneva.

Numerous workers have reported data which show that eating utensils are important in the transfer of respiratory and saliva-borne infections.

Since the basic danger lies in the incomplete removal of consumer-contamination, the present study has been conducted to ascertain the validity of employing *Streptococcus salivarius* as an indicator-organism of mouth contamination. The closed lips of 100 individuals were wiped with sterile cotton swabs. To determine whether the organisms from the lips and mouth are deposited on drinking glasses, each

individual was given a drink of sterile fruit juice from a sterile glass. The rim of the glass was then swabbed with a sterile swab. The swabs were incubated for 12 hours at 38°C. in enrichment broth and then plated in 8 per cent horse-blood veal infusion agar and incubated for 24 hours at 37°C. Typical streptococcal colonies exhibiting *alpha* to *gamma* hemolysis were picked and their fermentative reactions studied.

Streptococcus salivarius was recovered from the lips and drinking glass of every individual.

THE ETIOLOGY OF TUBERCULOSIS OF FISH. *J. A. Baker*, N. Y. State Veterinary College, Ithaca.

ON THE NATURE OF ADAPTIVE ENZYMES. *Otto Rahn*, N. Y. State College of Agriculture, Ithaca.

SHOCK DISEASE. *Carl L. Larson*, University of Rochester, Rochester.

A STRAIN OF ENTEROCOCCUS (STREPTOCOCCUS FECALIS) GIVING CROSS-REACTIONS WITH PNEUMOCOCCUS TYPE III ANTISERUM, ISOLATED FROM THE BLOOD STREAM OF A PATIENT. *E. Witebsky, J. MacCallum, E. Neter and C. Richbart*, The Buffalo General Hospital, Buffalo.

The strain of enterococcus under investigation was isolated from a patient with septicemia following abortion. It grew well on blood agar plates, causing greenish discoloration. It produced in 1 per cent glucose broth a pH of 4.5; it caused acid production, reduction, and clot formation in litmus milk, reduced methylene blue, fermented aesculin, produced acid from lactose, salicin, mannitol. It grew in 6.5 per cent NaCl-broth, was bile-insoluble, and grew in broth containing optochin in a concentration of 1:100,000. It was relatively heat-resistant. The supernate of the broth culture gave a definite reaction with Pneumococcus Type III antiserum, although the precipitation occurred somewhat more slowly and was somewhat less intensive than with Pneumococcus Type III. The suspension of the strain was agglutinated by Pneumococcus Type III antiserum. Although the strain failed to kill mice upon intraperitoneal injection, it could be recovered from the heart's blood. Smears of the peritoneal exudate revealed encapsulated diplococci; the supernate giving precipitation with Pneumococcus Type III serum. Other pneumococcal antisera (including Type VIII) failed to give any reaction with either the culture or the peritoneal exudate. The cross reaction with this particular strain was constantly found for about ten weeks; subsequent subcultures, however, lacked this property.

THE ACTION OF SULFANILAMIDE ON MENINGOCOCCI IN VIVO AND IN VITRO. *Erwin Neter*, Children's Hospital and University of Buffalo, Buffalo.

At the Children's Hospital two cases of meningococcal meningitis were treated with sulfanilamide alone. One patient was a baby ten weeks old who was suffering from a recrudescence of meningococcal meningitis. After the intraspinal injection of sulfanilamide, the spinal fluid became sterile in 72 hours and the patient made an uneventful recovery. The spinal fluid of the second patient became sterile 24 hours after the administration of large doses of sulfanilamide by mouth.

The action of the sulfanilamide upon meningococci in the spinal fluid from patients with meningococcal meningitis was studied. It was found (1) that sulfanilamide inhibited the growth of meningococci in spinal fluid; (2) that

previous treatment of meningococci with sulfanilamide was followed by a retardation of the growth rate on subculture or by a loss of viability. These effects of sulfanilamide were also obtained in the absence of leucocytes.

THE BACTERIOSTATIC EFFECT OF SULFANILAMIDE, DISULON, AND NEOPRONTOSIL IN VITRO ON ACID-FAST BACILLI. *C. M. Carpenter and L. L. Gibbons*, University of Rochester School of Medicine and Dentistry, Rochester.

Thirty strains of acid-fast bacilli were exposed *in vitro* to 1:10,000 concentrations of sulfanilamide, Disulon, and Neoprontosil. This procedure was repeated at least 5 times with each strain. A number of the strains were exposed in the same manner to 1:5,000 and 1:2,500 concentrations of each compound.

Twenty-one of the strains were chromogenic. Fourteen were isolated from cases of cutaneous leprosy, and 7 from miscellaneous sources. Of the 9 non-chromogenic strains, 5 were from lepromata, and 1 from sputum; 3 were *Mycobacterium tuberculosis*, avian and human strains.

Exposure to sulfanilamide and to Disulon inhibited completely or partially the growth of 8 strains of so-called *Mycobacterium leprae*, 1 strain of *Mycobacterium leprae-murium*, and 1 unidentified strain isolated from sputum. Of these leprosy strains, 4 were isolated by Kedrowski, 3 in our laboratory, and 1 by Needham. No inhibition was noted in the growth of the strains of *Mycobacterium tuberculosis*, the so-called saprophytic chromogenic strains, and the 6 remaining cultures isslated from lepromata.

Concentrations of the drugs greater than 1:10,000 showed no further inhibition. Neoprontosil did not inhibit growth in any instance. No bactericidal effects were observed. The action of sulfanilamide and Disulon was entirely bacteriostatic.

RESISTANCE OF THE GONOCOCCUS IN VITRO TO GRADUALLY INCREASED CONCENTRATIONS OF SULFANILAMIDE. *R. A. Boak and C. M. Carpenter*, University of Rochester School of Medicine and Dentistry, Rochester.

Six strains of *Neisseria gonorrhoeae* were grown in blood-glucose-ascitic fluid broth containing gradually increased concentrations of sulfanilamide and tested daily for viability.

Two strains grew well in 0.01 per cent sulfanilamide on isolation, but failed to survive after 72 hours in 0.02 per cent. After daily transfers for 17 and 12 days, respectively, in 0.01 per cent sulfanilamide, both strains thrived in a 0.02 per cent concentration.

Three strains, which survived for only 24 hours in 0.02 per cent sulfanilamide, were grown for 35 days in 0.01 per cent and then exposed to concentrations of 0.02, 0.03, 0.04, 0.05, and 0.06 per cent of the compound. Each continued to grow in the first three concentrations. In 0.05 and 0.06 per cent, a few organisms from 2 strains remained viable for only 24 hours, while the third strain did not survive.

The sixth strain, recovered from a patient after extensive sulfanilamide treatment, grew luxuriantly in 0.04 per cent when isolated. It required cultivation in 0.02 per cent for 91 days, with daily transfer, before it grew satisfactorily in 0.08 per cent sulfanilamide. A control culture, cultivated without sulfanilamide for the same period of time, did not survive 0.08 per cent for 24 hours.

EASTERN NEW YORK BRANCH

DIVISION OF LABORATORIES AND RESEARCH, STATE DEPARTMENT OF HEALTH
DECEMBER 2, 1938

SOME EFFECTS OF LOW VELOCITY ELECTRONS ON MICROÖRGANISMS. *Caryl P. Haskins*, Union College, Schenectady, New York.

An instrument has been constructed to study the effects of beams of low-velocity electrons of high homogeneity in density and velocity upon various microörganisms. All work is of necessity done in high vacuum. The work is of interest for a number of reasons. The beam acts essentially as a blunt-edged probe wherewith to explore the organism. It may be hoped to shed some light on the quantitative nature of the action of x-rays on living single cells, since the absorbed electrons can be made equal in number and range to the average range and density of the released secondary electrons in tissue when bombarded by x-rays of any desired wavelength over a wide range. It can also be used in investigations of the killing "sensitive volume" concept, and in a study of the mechanism of mutations under ionizing radiations. Work so far has been done with spores of Ascomycete fungi of the genera *Penicillium* and *Aspergillus*, which show good resistance to vacuum killing. Some mutations have been produced in *Aspergillus*, which are shown. It is expected to extend the work to both larger and smaller single-celled organisms, and to viruses.

A STUDY OF PNEUMOCOCCUS TYPE DIFFERENTIATION IN A GENERAL HOSPITAL. *Arthur W. Wright*. Department of Bacteriology, Albany Medical College, Union University, Albany, New York.

During a one-year period, 562 specimens of sputum for pneumococcus type differentiation were received in the laboratory of the Albany Hospital. These specimens came from 300 patients whose ages varied from 17 months to 79 years. Clinically, 93 of the patients suffered from lobar pneumonia, 96 from bronchopneumonia, 2 from empyema, one from meningitis, while 108 had no clinical evidence of pneumonia.

Lobar pneumonia was most common in the fourth, fifth and sixth decades, 49 of the 93 cases falling into these age groups. The predominating pneumococcus types found were I, III, IV, VIII, XII, V and XIV, in that order. These strains were incitants in 62 cases. In 10 cases pneumococci could not be found but other microörganisms, chiefly *Streptococcus hemolyticus*, were isolated. These may have been the inciting agents. Forty-four patients with pneumococcus lobar pneumonia were treated with specific antiserum. This group is too small to use as a basis for an analysis of results.

Of the patients with pneumococcus lobar pneumonia 17 had septicemia, as follows: Type I, 3 cases; Type III, 4 cases; Type XII, 4 cases; Type VIII, 2 cases; and Type IV, V, IX and XIV, one case each. Serum treatment was administered to the patients with Type I and Type V pneumococcus pneumonia and to one of the patients with Type XII pneumococcus infection. Two patients, one who had received Type V antiserum and the other Type XII antiserum, recovered. All others died.

A STUDY OF THE APPEARANCE OF SERUM AGGLUTININS AND POSITIVE S.S.S. SKIN REACTIONS IN PATIENTS WITH

PNEUMOCOCCUS PNEUMONIA—A PRELIMINARY REPORT. *Mabel S. Ingalls*, Department of Bacteriology, Albany Medical College, Union University, Albany, New York.

Sixteen of seventeen serum-treated pneumonia cases showed a strong titer of agglutinins in their blood, after administration of antiserum. The seventeenth showed no agglutinins and died. Of the 16 with agglutinins, 3 also died. On autopsy all 3 were found to have had complications not due to pneumococci.

Nine of the above cases were also tested for skin reactivity to the carbohydrate of the causative pneumococci. Seven gave positive reactions, one was negative, despite a strong agglutination titer and died, and one never gave a frankly positive reaction, lost his agglutinin titer after 4 days and ran a temperature of 103°.

The tests for the presence of agglutinin in the patient's serum were carried out by the slide agglutination method of Bullowa.[1] The tests for skin reactivity to the specific carbohydrate were carried out, according to the technique of Francis,[2] with purified carbohydrates from the Division of Laboratories and Research of the N. Y. State Department of Health.

From these few results it would seem the agglutination test is of value in indicating the concentration of antibodies in the patient's blood.

The carbohydrate skin reaction is valuable in showing there are no serious complications and predicting recovery.

A PRELIMINARY REPORT ON THE CHEMOTHERAPEUTIC ACTION OF 2-(P-AMINOBENZENESULFONAMIDO)PYRIDINE, M. & B. 693, ON TYPE-III PNEUMOCOCCAL INFECTIONS IN MICE. *John K. Miller*, Division of Laboratories and Research, New York State Department of Health, Albany.

The blood concentration attained one and two hours after oral administration of 10-, 20-, or 40-mg. doses of M. & B. 693 to 20-gram mice was the same—from 22.5 to 25 mg. per cent of free M. & B. 693. Subcutaneous doses of 20 and 40 mg. produced a concentration of 10 and 20 mg. per cent, respectively. The free M. & B. 693 in the viscera was, in general, from 75 to 90 per cent of the blood concentration, but in the liver only 25 per cent.

The effect of M. & B. 693 and of M. & B. 693 combined with serum therapy on pneumococcal infection in 20-gram mice was studied with 10,000 M.L.D. of a stock mouse-passage strain of type-III pneumococcus. Ten-, 20-, or 40-mg. subcutaneous doses of M. & B. 693 alone or with one dose of specific antiserum that by itself afforded 20-per-cent survival were given immediately and at 7, 24, 48, 72, and 96 hours after inoculation of pneumococci to groups of ten mice. The combined therapy was definitely the more effective.

EFFECT OF KETENE ON DIPHTHERIC TOXIN. *L. W. Hyman*, Division of Laboratories and Research, New York State Department of Health, Albany.

The study of the effect of ketene on crude diphtheric toxin was undertaken to ascertain the extent of acetylation of the amino group and its possible significance in the formation of toxoid. The ketene was prepared according to the method of Hurd and Cochran (1923). To 100 ml. of toxin, 45 grams of sodium acetate were added and the mixture cooled to 5°C. On introduc-

[1] N. Y. State Jour. Medicine, **37**, Apr. 15, 1937.

[2] J. Ex. Medicine, **57**, 4, 617, 1933.

ing ketene, the pH was lowered; normal sodium hydroxide was added during the acetylation to maintain the desired pH. The solutions were dialyzed against running water and then either adsorbed on calcium phosphate and dissolved in ammonium citrate or concentrated by ultrafiltration. The acetylations were done at pH 7.0, 8.0, and 8.2. The reduction of amino nitrogen ranged from 40 to 100 per cent; with a reduction of over 50 per cent, the Lf value and the number of M.R.D. per milliliter decreased. The acetylated toxins had little or no antigenicity. One toxin which had 1,000,000 M.R.D. per milliliter and an Lf of 18.8 in twenty-six minutes was treated with ketene at pH 8.0 for one hour. The amino nitrogen was reduced 93 per cent, the number of M.R.D. to 500 per milliliter, and the Lf to 13.4, with the time of flocculation increased to four hours.

THE EFFECT OF COLCHICINE ON TISSUE CULTURES OF TADPOLE HEART. *Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

Although colchicine has been reported as relatively nontoxic for frogs, tadpole cardiac tissue proved susceptible. Fragments failed to show migration and growth when explanted into a medium containing colchicine in a concentration of 1 part per 10,000,000. A concentration of 1:100,000,000 did not inhibit growth.

When one loopful of colchicine, diluted 1:50,000 in Tyrode's solution, was allowed to act for from six to eight hours on cultures in which growth had become established, no mitoses were seen in stages beyond metaphase. Many mitoses were abnormal. A marked increase was noted in the number of dividing cells in comparison with untreated control cultures.

A comparable accumulation of mitoses was seen in cultures treated with diphtheric toxin for twenty-four hours before the application of colchicine. This suggests that the preliminary treatment with toxin had not caused a cessation of cellular activity.

A COLLODION SAC FOR USE IN ANIMAL EXPERIMENTATION *Albert H Harris*, Division of Laboratories and Research, New York State Department of Health, Albany

The sacs described represent a radical departure from those of Gates (1921) although many of his technical details have been adopted. Stainless steel-wire gauze curved as a cylinder is closed at both ends with molded carnauba wax and a glass tube is passed through one end. Both ends of the sac and the line of approximation of the gauze are covered with alcohol-ether collodion which is allowed to dry for several hours. The sac is dipped in the percentage of acetic-acid collodion required in the given experiment, allowed to drain for a specified time (the collodion which collects at the bottom on the inside is aspirated), and then plunged into filtered tap water. All of the solvent is washed out in periodic changes of the water. The rate of flow of water is measured. The sac is sterilized for about eighteen hours in a solution of 1 part of B.K. (hypochlorite solution) in 100 parts of distilled water and then washed in a large volume of sterile distilled water. The sac is filled by a Pasteur pipette and sealed with paraffin and collodion. At the completion of the *in vivo* experiment, the sac is opened by thrusting a hot wire through the collodion and paraffin seal, and the contents are removed by a Pasteur pipette.

OHIO BRANCH

DECEMBER 3, 1938

A SIMPLIFIED APPARATUS FOR THE PRESERVATION OF BACTERIAL CULTURES IN THE DRIED STATE. *Frank J. Grabill and Merlin L. Cooper*, Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati.

A simple vacuum apparatus was designed to freeze and dry 32 bacterial cultures simultaneously. The apparatus consists of a vacuum pump, two Wolff bottles, attachments for 32 glass ampules of culture and a chemical desiccant, "Drierite."

The principle involved is that of reducing the vapor pressure of the system to the extent that the moisture will evaporate from the surface of the bacterial cultures with sufficient rapidity to freeze them. Once frozen, the water sublimes from the frozen culture to the desiccant, the rate being sufficient to retain the cultures in the frozen state throughout the desiccation period. The ampules containing the bacterial cultures are blown from ordinary 9 mm. glass tubing. After the dehydration of the cultures, the ampules while still attached to the vacuum apparatus are sealed with a suitable blow torch.

All members of the *Salmonella*, *Eberthella* and *Shigella* groups so preserved have retained their viability and cultural and biochemical characteristics, without any detectable changes, over a period of six months. Further studies and observations are in progress.

The simplicity of the apparatus makes low-temperature desiccation of heat-labile substances available to the average laboratory.

AN ACCESSORY FACTOR REQUIRED FOR THE SYNTHESIS OF POLYSACCHARIDE BY LEUCONOSTOC. *Warner Carlson*[1] *and G. L. Stahly*, Department of Bacteriology, Ohio State University, Columbus.

Studies were made of the mode of action and chemical nature of the raw sugar extract reported by Carruthers and Cooper as stimulating the synthesis of the polysaccharide, dextran, by *Leuconostoc mesenteroides* and *L. dextranicum* in media containing chemically pure sucrose.

Determinations of bacterial numbers, sucrose titers, dextran yields and hydrogen-ion changes in C. P. Sucrose media with and without added raw sugar extract indicated that the stimulating material should be referred to as an "accessory" rather than a "growth" factor, since it caused a marked stimulation of the rate of dextran synthesis without a corresponding significant increase in the observed number of bacteria per cc. Three types of *Leuconostoc* could be distinguished on the basis of ability to synthesize dextran in a medium containing C. P. sucrose, phosphate and peptone: (a) type 1, which did not require the presence of the factor to form approximately the theoretical amount (50 per cent of the sucrose in the medium) of dextran in 48 to 72 hours time; (b) type 2, which could also synthesize the maximum amount of dextran in the absence of any added factor, but only after about 10 days incubation; and (c) type 3, which gave yields of dextran of only a few per cent of the

[1] H. A. Toulmin, Jr. Research Fellow.

theoretical, regardless of the length of the incubation period. Addition of the factor to the medium allowed all three types to form approximately the theoretical amount of dextran in 24 to 36 hours.

The factor was extracted by alcohol from some unrefined cane sugars, but it was not found in any of the unrefined beet sugars tested. The accessory factor was also demonstrated in the sterile, cell-free filtrates of broth cultures of some strains of *Staphylococcus aureus*, and of C. P. sucrose cultures of type 1 *Leuconostoc*.

Tests on highly active alcoholic extracts of raw sugar demonstrated that the factor is: (1) not a protein; (2) soluble in methyl alcohol, ethyl alcohol and pyridine; (3) insoluble in acetone, ether, dioxane, benzene and ethyl acetate; (4) not adsorbed by activated charcoal; (5) adsorbed by aluminum gels at pH 10, and eluted at approximately pH 4; and (6) resistant to 2 hours autoclaving at 120°C. at pH 4.2, but destroyed under the same conditions at pH 8.5.

DILUTED AND UNDILUTED DIPHTHERIA TOXOID AS IMMUNIZING AGENTS IN MAN. *Hortense Schmitz*, Department of Hygiene and Bacteriology, Western Reserve University, Cleveland.

A COMPARATIVE STUDY OF BACTERIOLOGICAL EXAMINATIONS OF RESTAURANT UTENSILS. *Otto P. Behrer and Wm. M. Moody*, Cincinnati Department of Health, Cincinnati.

Three bacteriological methods of testing restaurant eating utensils are described and evaluated: the direct-culture, moist-swab and the wet-swab methods proposed by Fellers, Levine and Harvey. The direct-culture method consisted in collecting knife, fork, spoon, glass and plate in sterile towels and bringing them into the laboratory. Forks, spoons and knives were immersed in melted nutrient agar in sterile petri dishes. The rim of the glass was also immersed in agar and the inside washed with 10 cc. of sterile water of which 1 cc. was plated. The same procedure of washing and plating was followed with the plates. The moist-swab method consists in adding 0.4 cc. of water to the tube containing the swab and autoclaving. In practice the swab is rubbed over the surface to be examined, replaced in the tube and submitted to the laboratory. It is now transferred to a tube of sterile water (2.5 cc.) vigorously shaken and the entire liquid plated in melted agar. The wet-swab method uses 1 tube containing the 2.5 cc. of sterile water with swab, and is used in the same manner as the moist-swab.

In actual practice the moist-swab method was found more practical and is recommended as the method of choice, both because of its adaptability to field service and because of the results obtained.

HETEROPHILE ANTIGENICITY OF BRUCELLA ABORTUS. *D. Frank Holtman*, Department of Bacteriology, Ohio State University, Columbus.

Previous investigators have considered *Brucella abortus* to be free of Forssman heterophile antigen. However, evidence obtained during the present experimentation shows that certain strains of this bacterium can stimulate in rabbits the formation of antibodies capable of agglutinating or hemolyzing sheep red blood cells. Two cultures of *B. abortus*, isolated from supraspinous bursitis in horses, possessed this property even after 100 transfers on an agar medium free of heterophile antigen. Two cultures iso-

lated from cattle failed to demonstrate heterospecificity, although a third culture of bovine origin, after several years of growth on horse serum agar, was able to induce heterophile antibody formation in rabbits. Since the horse provides a rich source of Forssman antigen, an agent lacking in cattle, it seems probable that the heterospecificity of the bacteria was acquired, not only from the living animal, but also from culture media incorporating its serum.

SYSTEMIC TORULOSIS WITH CASE REPORT AND WITH REFERENCE TO EARLY CASES SIMULATING HODGKIN'S DISEASE. *Thomas L. Ramsey*, St. Vincent's Hospital, Toledo.

THE SPECIFICITY OF THE KERATINS. *L. Pillemer and E. E. Ecker*, Institute of Pathology, Western Reserve University, Cleveland.

A BACTERIOLOGICAL AND SEROLOGICAL STUDY OF 245 INFANTS AND YOUNG CHILDREN WITH ACUTE GASTRO-ENTERITIS. *Merlin L. Cooper*, with the assistance of *H. M. Keller, J. B. Milliken, B. Johnson, H. F. Marsh, F. J. Grabill and G. W. Thomas*, Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati.

During the summer of 1938, 796 stools from 245 infants and young children with acute diarrhea were cultured for *Shigella paradysenteriae*. Each stool was streaked on four plates each of Endo's medium, eosin methylene-blue medium, sodium desoxycholate citrate medium and MacConkey's medium. Suspicious colonies were picked and their cultural characteristics determined in suitable carbohydrate media, gelatin and citrate agar. Motility observations were made after 4 and 24 hours' incubation. Forty-one per cent of these patients were found to have *Shigella paradysenteriae* in one or more of their stools.

In the order of decreasing efficiency the four media were as follows: sodium desoxycholate citrate, MacConkey's, eosin methylene-blue, and Endo's.

Three different fermentative types of *Shigella paradysenteriae* were isolated, differing in carbohydrate reactions.

The blood sera of 70 patients were tested for agglutinins for antigens prepared from *Shigella paradysenteriae* isolated from earlier patients and 22 were found positive, 12 slightly positive, and 36 negative.

Agglutinating antisera had also been prepared prior to this summer for 4 of our 7 antigens. The *Shigella paradysenteriae* isolated from 90 of our patients during this study were tested for their agglutinability by these sera. Fifty-five were agglutinated significantly, 12 were doubtful, and 22 were negative.

FURTHER EXPERIMENTS ON THE WASHING OF HEMOPHILUS PERTUSSIS VACCINE. *J. A. Toomey and William Takacs*, Department of Pediatrics, Western Reserve University, Cleveland.

THE GROWTH REQUIREMENTS OF BACILLUS ANTHRACIS. *Maurice Landy*, Research Division, S.M.A. Corporation, Cleveland.

It has been found that, although Knight observed that his "staphylococcus factor" of yeast stimulated growth of *Bacillus anthracis* and subsequently discovered that in his yeast concentrate nicotinic acid and thiamin were the biological factors essential for the growth of *Staphylococcus aureus*,

it is not these two vitamins but still another factor in yeast that is essential for the growth of *B. anthracis*.

The *B. anthracis* growth factor is not replaced by thiamin, nicotinic amide, beta alanine, pimelic acid, glutathione, betaine, riboflavin, glucosamine, vitamin B_6, or the acidic ether extract of yeast, either singly or in various combinations.

Meat extract, liver extract and yeast are good sources of the growth factor.

Two concentrates of the *B. anthracis* factor prepared from yeast exhibited growth stimulation at levels of 1 and 30 micrograms, respectively.

High vacuum distillation of a potent concentrate at temperatures from 120° to 160°C. at 0.002 mm. mercury results in the activity remaining in the residue.

The growth factor is resistant to autoclaving, to acid and alkali, and to light, is adsorbed on norite charcoal at pH 1.4, is soluble in water and aqueous alcohol, and is insoluble in acetone, ether and chloroform.

CENTRAL PENNSYLVANIA BRANCH

STATE COLLEGE, PA., NOVEMBER 26, 1938

COMPARATIVE STUDY OF THE INTRACUTANEOUS INJECTION OF P.P.D. AND THE VOLLMER PATCH TEST FOR THE DETECTION OF TUBERCULOSIS. *John W. Rice*, Bucknell University.

Due to reluctance on the part of many college students to submit to the use of the hypodermic needle, it was decided to study the patch test devised by Vollmer for the testing of young children.

A group of 265 students were tested by intracutaneous injection of P.P.D. in standard dose and simultaneously with the Vollmer Patch Test. To summarize, 52.8% of the group were negative to both tests; 23% were negative to the Vollmer test but showed a one plus reaction to P.P.D.; 13.6% showed agreement in one plus reactions while in the group of fairly strong reactions to P.P.D., 0.75% were negative; 2.64% were faintly positive; and 1.6% were definitely positive to the Vollmer test. A total of 5.3% showed stronger reactions to the Vollmer test than to P.P.D. Delayed reactions were quite common in the case of the Vollmer test.

The relative high number of negative reactions together with the delayed response of the Vollmer Patch Test would seem to indicate that this test is not applicable to the college age group.

STUDIES OF CACAO FERMENTATION. *S. Hoynak, T. S. Polansky and R. W. Stone*, Pennsylvania State College.

A study has been made of the microflora of cacao beans, together with some resulting chemical changes produced by the microorganisms during fermentation. Counts of viable organisms were made daily during the fermentation period. Relative percentages of types, the stage of their appearance in the fermentation, and their isolation and identification were carried out.

During the initial or alcoholic stage of the fermentation, yeast-like organisms predominate. This is followed by an acetic and later a proteolytic stage. In both these latter stages gram-negative rods are most numerous. Utilization of reducing sugars, pH, and titratable acidity were followed. Determinations were made on fermenting beans at 25°C., 36°C., and 45°C. Under the experimental conditions, variations

in temperature generally resulted in a change of rate rather than in type of fermentation.

A Study of the Epidemiology of Tobacco Wildfire. *H. D. McAuliffe, M. A. Farrell, D. E. Haley and J. J. Reid,* The Pennsylvania Agricultural Experiment Station.

The epidemiology of tobacco wildfire has been the subject of a study carried out as a threefold program consisting of a general survey of the tobacco-growing regions of Pennsylvania, soil fertility experiments and laboratory investigations.

It has been found that the incidence of tobacco wildfire cannot be related to the incidence of *Phytomonas tabaca* inasmuch as the organism has been present in all tobacco examined, whether exhibiting lesions or free from all evidence of disease.

On the other hand, normal tobacco of the cigar-leaf filler type has been found to be very resistant to infection of economic severity during the growing season. Normal tobacco contains a nitrogen-potassium ratio of somewhat less than one. Tobacco with a ratio greater than one has been found relatively susceptible during the growing season.

During the ripening period, tobacco may become susceptible regardless of previous nutrition if any significant amount of nitrogen is taken up by the plant. This susceptibility has been found to be increased by topping and suckering, practices which take from the plant the last possibility of the normal use of nitrogen at this period in the proliferation of new tissue.

The conclusion is drawn that the control of tobacco wildfire is largely a matter of plant nutrition.

The Relation of Delayed Ammonification and Nitrification to Leaf Spots of Tobacco. *J. Naghski, H. D. McAuliffe, D. E. Haley and J. J. Reid,* The Pennsylvania Agricultural Experiment Station.

Susceptibility of tobacco to leaf spot diseases during the ripening period has been found to be associated with a significant nitrogen uptake at this stage.

The presence of nitrates in the soil in excessive amounts during the ripening period has been shown to be due to delayed ammonification and nitrification of the organic nitrogen applied in the spring in some instances, and in others to delayed nitrification of ammonium salts which have probably remained for extensive periods in some relatively insoluble form such as ammonium magnesium phosphate.

Delay in ammonification and nitrification is usually associated with lack of sufficient rainfall in the growing season. If this condition is followed by rain during the ripening period an excessive amount of ammonification and nitrification takes place, the normal resistance of tobacco to leaf spot diseases is lost and a serious outbreak of disease occurs.

The conclusion is drawn from these studies that it is unsafe to incorporate large quantities of complex organic nitrogen in a soil to be used for tobacco. From this standpoint tobacco should not follow a legume in the rotation nor should any animal manure be applied to the crop that has not been previously rotted.

A Cultural and Serological Study of Phytomonas tabaca and Certain Related Forms. *J. J. Reid, J. Naghski, R. G. Harris and H. D. McAuliffe,* The Pennsylvania Agricultural Experiment Station.

A study has been made of a number of strains of *Phytomonas tabaca* and strains of a few other species of the genus *Phytomonas* associated with leaf spot diseases. These have been investigated physiologically and serologically and compared with known strains of *Pseudomonas fluorescens* and with more than 2000 isolations of *P. fluorescens* from the leaves of normal, healthy tobacco and clover.

It has been found possible through culture in unheated plant extracts high in nitrogen to bring about a physiological adaptation of *Pseudomas fluorescens* that is physiologically and serologically identical with strains of *Phytomonas tabaca*.

Such adaptation may be induced rapidly in the laboratory and may be as rapidly destroyed by frequent passage through other laboratory media.

As a result of this study the conclusion has been drawn that *Phytomonas tabaca* represents merely a transitory physiological adaptation of *Pseudomonas fluorescens*.

The work with the other representatives of *Phytomonas* associated with leaf spots while not as conclusive, suggests that other members of the genus are also temporary physiological adaptations of *Pseudomonas fluorescens*.

EASTERN MISSOURI BRANCH

NOVEMBER 8, 1938

STUDIES ON RABIES. 1. COMPARISON OF GUINEA PIG AND MOUSE INOCULATION METHODS. *S. Edward Sulkin and Joseph C. Willett*, Laboratories of the St. Louis Health Division, St. Louis, Missouri.

A simple technique for the early diagnosis of rabies by mouse inoculation is described as a convenient means of verifying the routine microscopic (Negri body) method. The results of the experiments presented show that (1) the white mouse is the animal of choice in the laboratory diagnosis of rabies, Swiss mice being of no practical advantage; (2) that the demonstration of Negri bodies in the test animal is the only dependable criterion for a positive diagnosis since clinical manifestations are frequently too variable and indefinite to be of any value; (3) that it is practical to attempt demonstration of Negri bodies by sacrificing mice on the 8th or 9th day after the inoculation; and (4) that the mouse inoculation test offers a more expedient and cheaper means of diagnosis than the guinea pig method.

STUDIES ON RABIES. 2. DEMONSTRATION OF RABIES VIRUS IN GROSSLY DECOMPOSED ANIMAL BRAINS. *S. Edward Sulkin and Nathan Nagle*, Laboratories of the St. Louis Health Division, St. Louis, Missouri.

Putrified brain tissue is usually difficult material in which to demonstrate Negri bodies and for this reason the diagnosis of a decomposed brain by microscopic examination is unreliable.

Experiments are presented describing a simple procedure for demonstrating rabies virus in grossly decomposed animal brains. In the preliminary experiments several bactericidal agents including merthiolate, glycerol, phenol, and ether were used in an effort to find one which would conveniently sterilize the heavily infected inoculum without appreciably effecting the virus. Ether used according to the method suggested by Dr. John R. Paul (J. Bact., *35*, 493,

1938) and others proved to be the most desirable agent. It was found that ether in final concentration of 10% exerts a definite bactericidal effect upon contaminated dog brains after exposure for 2 hours at 4°C. Exposure to this concentration of ether for 18 hours at 4°C. had no effect upon the virulence of two strains of street virus tested. The experiments were conducted with the view of establishing a simple and practical method for treating contaminated brains before intracerebral injection into animals.

PROPHYLAXIS OF TETANUS. *Philip Varney*, Department of Bacteriology, School of Medicine, Washington University, St. Louis.

REPORT ON THE HOT SPRINGS, ARKANSAS, CONFERENCE ON SYPHILIS. *R. B. H. Gradwohl*, Gradwohl Laboratories, St. Louis.

227-244 omitted in numbering.